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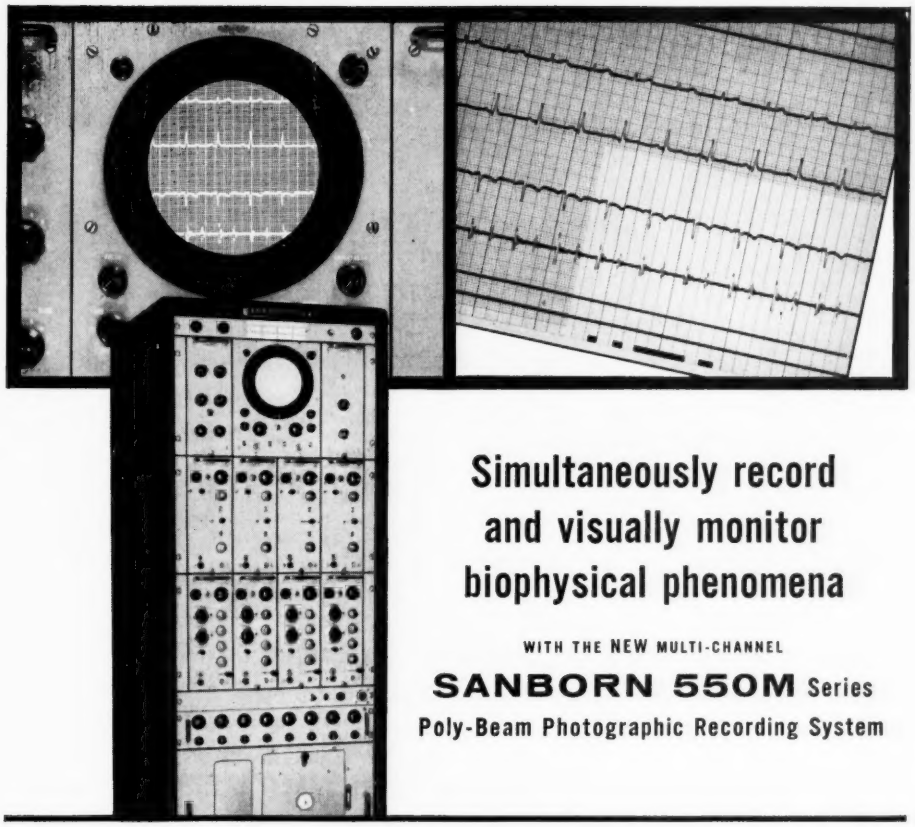

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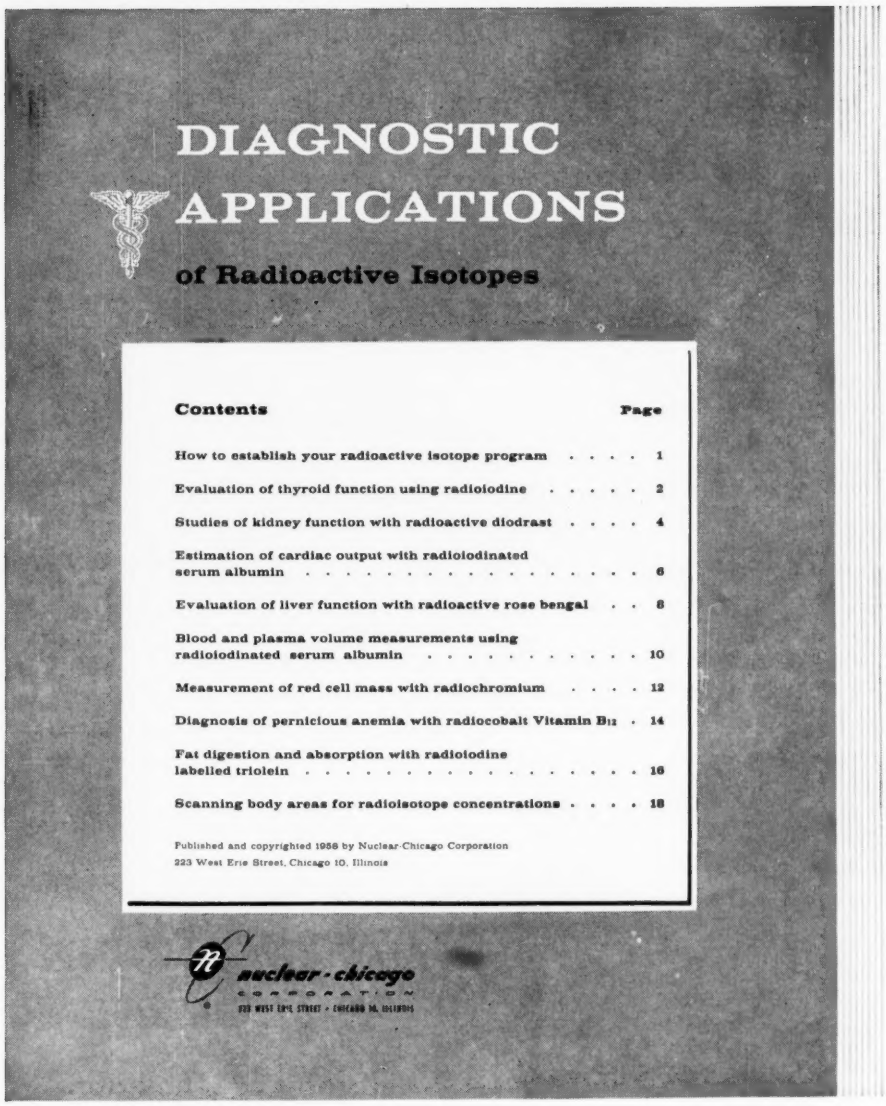
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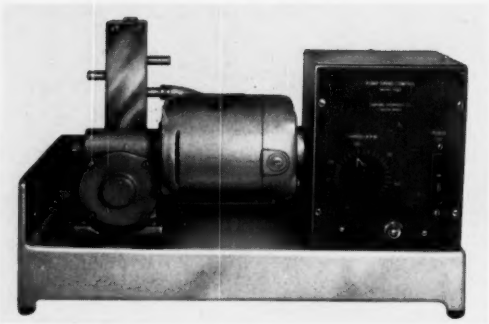

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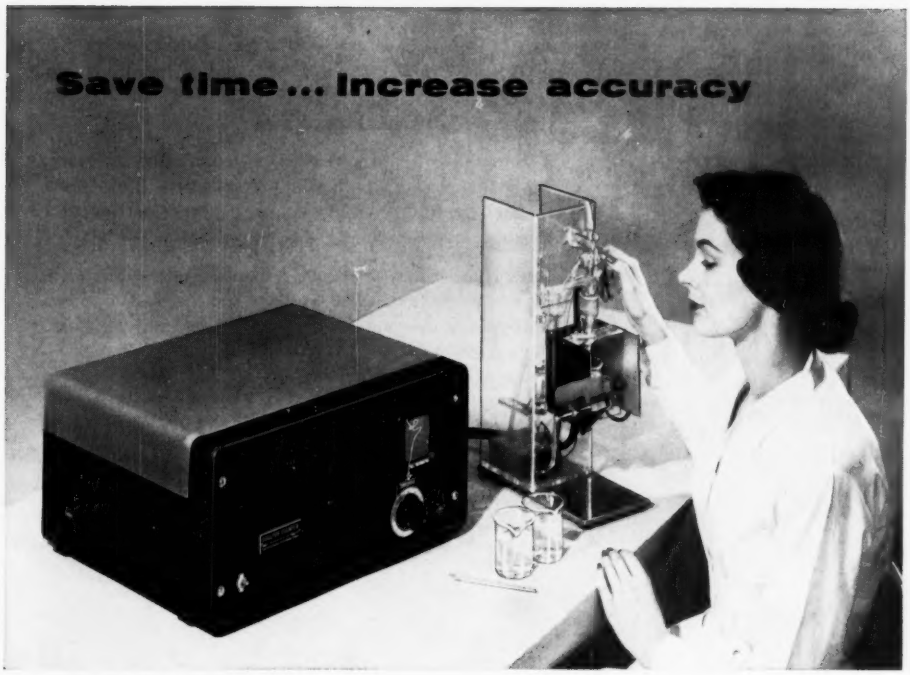

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HISTAMINE AND 5-HYDROXYTRYPTAMINE IN THE BLOOD OF INFANTS AND CHILDREN

By ROSS G. MITCHELL and ROSEMARY CASS *

(From the Departments of Child Health and of Pharmacology and Therapeutics, University of St. Andrews, Dundee, Scotland)

(Submitted for publication October 6, 1958; accepted November 6, 1958)

Evidence from a number of sources indicates that the amount of histamine in the blood of healthy adult human beings is usually within the range 0.01 to 0.08 μg. per ml. (1) and that nearly all of it is in the basophil and eosinophil leukocytes (2, 3). The little information available about the blood of children suggests that the total content of histamine is similar to that of adult blood. Thus, Maas, Fehmers and Strengers (4) estimated the histamine content of the blood of 51 normal children. Several determinations were made on each child and the average values recorded; all except two fell within the adult range, although individual values were more variable. Unfortunately, these workers did not relate the values obtained to the numbers of basophils and eosinophils in the blood, nor did they determine whether any of the histamine was in the plasma. Little if any histamine is normally present in the plasma of healthy adults (5), but the report by Pettay (6) that the plasma of umbilical cord blood contained 0.06 to 0.20 μg. of histamine per ml. suggested that the blood in early life might differ in this respect from adult blood. The present investigation was undertaken to determine the amount of histamine in the blood at birth and in childhood and its distribution among the various elements of the blood.

There is usually a numerical relationship between the basophils and eosinophils in the blood (7, 8), although not a close one, and in quantitative studies it has proved difficult to determine the distribution of histamine between the two types of cell (3). In an attempt to shed further light on this problem, a study was made of children who had abnormally large numbers of these cells in the circulation or in whose blood there was a wide dissociation between the numbers of basophils and eosinophils.

There have been few reports on the 5-hydroxytryptamine (HT) content of the blood in infancy and childhood. According to Erspamer (9), the serum of newborn infants contains only a small quantity of HT (0.07 μg. per ml.). In contrast, higher values have been recorded in older children by Pare, Sandler and Stacey (10), who found the mean value in the serum of 15 children awaiting tonsillectomy to be 0.124 μg. per ml. However, since HT is contained almost solely in the platelets, values obtained from serum are likely to be very variable, depending on the extent to which HT has been lost from the platelets during clotting. In the present study, HT was estimated in the platelets of the infants and children, and the plasma was also examined to detect any HT which might have escaped during collection or separation of the blood.

MATERIAL AND METHODS

Blood from 11 mature newborn infants was obtained from the umbilical vein before division of the umbilical cord, using a 30 ml. siliconed glass syringe. Five ml. of the blood was immediately added to 1 ml. of 3.8 per cent sodium citrate in a siliconed tube for determination of HT. Six ml. was added to 0.1 ml. 3.8 per cent sodium citrate in a siliconed tube and immediately centrifuged for five minutes, after which a measured quantity of the plasma was added by siliconed pipette to 5 ml. of 10 per cent trichloracetic acid for determination of plasma histamine. While this sample was being centrifuged, counting pipettes were filled directly from the blood in the syringe for enumeration of basophils, eosinophils and platelets. Next, two samples of 5 ml. blood were taken in siliconed pipettes and each added to 10 ml. of 10 per cent trichloracetic acid for duplicate determinations of total blood histamine. The remaining blood was used for the determination of the potassium content of the plasma. The whole procedure of partitioning the blood between the various containers was accomplished rapidly in the labor room and was completed within a few minutes of birth.

Blood from 16 children aged between one and 12 years was drawn from the anterior cubital vein, the routine being the same as for umbilical venous blood. Blood from 10 infants under one year old was drawn from the in-

* Present address: Laboratory of Chemical Pharmacology, National Heart Institute, Bethesda, Md.

ternal jugular vein, using a 10 ml. siliconed syringe since only about 6 ml. was required for HT determination and a platelet count. The children and infants were in hospital either for correction of an anatomical abnormality, such as inguinal hernia, or for investigation of some minor complaint for which no cause was found. None had a history of allergic conditions and all could reasonably be regarded as healthy children. In each case, venous blood was required for routine diagnostic purposes and blood for the investigation was taken at the same time to avoid an unnecessary venipuncture. Venous blood from six healthy adults was also examined to obtain an indication of the normal adult value for HT by our method.

The capacity of the blood platelets to take up HT was assessed by incubating a suspension of platelets for one hour at 37° C. in the presence of an excess of HT, using a suspension of the same platelets incubated with saline alone as the control. This test was carried out on two samples of umbilical venous blood and on two samples of venous blood from healthy adults.

Four children with eosinophilia were examined, eosinophilia being defined as more than 1,000 eosinophils per cu. mm. of blood. In two, the eosinophilia was associated with normal numbers of basophils, while in the other two there was also a basophilia. The first child (Case 1) was a boy of four years who had periorbital edema of six weeks' duration. Investigation revealed no cause for the eosinophilia and he had no other symptoms. The swelling gradually subsided but the eosinophil count remained high, though temporarily reduced by cortisone; one year later, the eosinophilia was still present.

The second child (Case 2) was a boy of five years, who had periorbital edema of three days' duration. He had a history of mild asthma and edema whenever he came in contact with cats. The swelling and eosinophilia subsided within a few days without specific treatment.

The third child with eosinophilia (Case 3) was a girl of six and one-half years with celiac disease, which responded to a diet without gluten. On admission, her blood contained large numbers of basophils and eosinophils, which increased still further during the next two months in spite of clinical improvement. A single dose of 200 mg. of cortisone only caused a small reduction in the numbers of basophils and eosinophils, and when she finally went home she still had a gross basophilia and eosinophilia. Her eight year old brother (Case 4) was also found to have a basophilia and eosinophilia. Full investigation of these two children, their parents, and their environment revealed no cause for the abnormal blood counts.

No case of basophilia without eosinophilia was encountered, but observations were made on one boy aged six years (Case 5), who had a low eosinophil count with a basophil count at the upper limit of normal. He had acute rheumatism of two weeks' duration, with signs of cardiac failure, the expected eosinopenia, but an unexpectedly high basophil count (110 per cu. mm.). In spite of treatment with sodium salicylate and cortisone, which resulted in a reduction in the number of basophils (to 25 per cu. mm.), there was no clinical improvement and he died five days after admission.

One further child, a girl aged 11 years with acute rheumatic carditis (Case 6), was studied throughout the course of her illness to determine whether the level of blood histamine bore the same relationship to the basophil and eosinophil counts as in health.

Histamine in blood was determined by Code's modification (11) of Barsoum and Gaddum's method (12). Histamine was extracted from plasma by the same method, using smaller quantities. Thus 2.5 to 3 ml. of plasma was added to 5 ml. of 10 per cent trichloracetic acid, filtered and washed with 5 ml. of 5 per cent trichloracetic acid. Three ml. of concentrated hydrochloric acid was added and the procedure continued as for whole blood. In seven recovery experiments, in which 0.05 or 0.1 μg. of histamine was added to each ml. of plasma, the mean percentage of the added histamine recovered was 85 ± 12.

For assay, which was carried out on a strip of guinea pig ileum suspended in atropinized Tyrode's solution, the dried residue was taken up to its original volume in Tyrode's solution diluted 3: 1 with distilled water, and neutralized with sodium hydroxide. Each sample was assayed by comparison with a standard solution of histamine acid phosphate, and evidence that the activity measured was due to histamine was obtained by frequent tests with mepyramine maleate (13). All values for blood histamine in this paper are mean values of duplicate samples and are expressed in terms of the free base.

The plasma potassium content of each sample of blood was estimated by flame photometry, since abnormally high levels of potassium might have interfered with the assay of histamine. In every instance the potassium was within the normal range. Basophils and eosinophils were counted by the method of Moore and James (14), with the modifications previously described (15, 16).

For the determination of HT in platelets and plasma, 5 ml. of blood with 1 ml. of 3.8 per cent sodium citrate was centrifuged at 1,000 rpm on a M.S.E. Major centrifuge (250 G) for 10 minutes. The supernatant, platelet-rich plasma was pipetted off and centrifuged at 3,500 rpm (3,000 G) for 20 minutes. The plasma was decanted off the sheet or "button" of platelets, and each was extracted with acetone, using 5 ml. for the platelets and four volumes for the plasma. The extracts were allowed to stand at room temperature for one hour and then centrifuged, and each supernatant decanted into a round-bottomed Quickfit flask. The residues were washed with 95 per cent acetone for 30 minutes and centrifuged, each supernatant being added to the contents of the corresponding Quickfit flask. Finally, the extracts were evaporated to dryness *in vacuo* at 37° C. external temperature, and stored at −17° C. until they could be assayed, which was nearly always accomplished within two days. All glassware used in the extraction before the addition of acetone was treated with silicone.

The assay was performed on the atropinized, estrous

TABLE I

Histamine and 5-hydroxytryptamine in the blood of healthy newborn infants *

No.	Sex	Basophils/ cu. mm.	Eosinophils/ cu. mm.	Histamine		Platelets	5-Hydroxytryptamine		
				Blood	Plasma		Buffy coat	Plasma	μg./10⁹ platelets†
				μg./ml.		thousands/ cu. mm.	μg./ml. of whole blood		
1	M	49	56	0.060	<0.020	171			
2	M	37	296	0.064		212	0	0	0
3	F	18	118	0.025		95	0.003	0	0.027
4	M	91	817	0.130		349	0	0	0
5	M	41	191	0.060		290	0	0	0
6	M	48	429	0.060	<0.010	435	0.027	0	0.060
7	F	36	469	0.048	<0.015	195	0.009	0	0.046
8	M	28	69	0.025	<0.008	160	0.015		0.094
9	M	66	351	0.075	<0.005	185	0.013	0.007	0.016
10	F	63	257	0.080	<0.005	258	0.019	0.014	0.129
11	F					207	0.014	0	0.067
Mean		47.7	305.3	0.063		233	0.012		0.053
S. D.		21.1	229.6	0.030		95.5	0.012		0.047

* Blood obtained from the umbilical vein at birth.

† In the last column, the HT content of the unwashed platelets (the "buffy coat" of the platelet-rich plasma) is added to the HT content of the plasma, and the total related to the platelet count made on whole blood.

uterus of the rat, and was repeated in the presence of the HT inhibitor lysergic acid diethylamide (10^{-7} Gm. per ml.). The HT content of each sample of blood was related to the volume of the sample and to the number of platelets. Since the HT in blood is derived solely from the platelets, the latter mode of expression is considered the more informative. Platelets were counted in whole blood by Lempert's method (17). Parallel counts on whole blood, and on separated platelet-rich plasma, when corrected for the difference in volume, consistently gave values within 10 per cent of one another, whereas counts on washed suspensions of platelets were always slightly lower. We therefore felt justified, in this comparative study, in relating the total HT from unwashed platelets and plasma to the numbers of platelets in whole blood, thus avoiding delay in starting the extractions.

RESULTS

Histamine

The mean content of histamine of 10 samples of umbilical venous blood was 0.063 ± 0.030 [1] μg. per ml. (range, 0.025 to 0.130 μg. per ml.). The mean basophil count was 47.7 ± 21.1 per cu. mm. (range, 18 to 91 basophils per cu. mm.), and the mean eosinophil count was 305.3 ± 229.6 per cu. mm. (range, 56 to 817 eosinophils per cu. mm.). In the six samples of plasma which were examined, no activity due to histamine was detected; the values are recorded as less than the smallest amount which could be measured by the method used, and this varied because of the varying sensitivity of different strips of guinea pig ileum (Table I).

In 15 healthy children, the mean value for the histamine content of whole blood was 0.049 ± 0.020 μg. per ml. (range, 0.020 to 0.080 μg. per ml.), the mean basophil count was 45.1 ± 20.8 per cu. mm. (range, 16 to 77 basophils per cu. mm.), and the mean eosinophil count was 256.2 ± 196.7 per cu. mm. (range, 47 to 664 eosinophils per cu. mm.). No histamine was detected in 12 of the 14 samples of plasma which were tested, but in two, very small amounts were found (Table II).

Figure 1 illustrates the relationship between the amount of histamine and the numbers of basophils and eosinophils in the 25 samples of blood.

The results obtained in the children with eosinophilia are shown in Table III. In Case 1, the initial eosinophil count and blood histamine level were abnormally high, and were still above normal 24 hours after starting cortisone (100 mg. daily by mouth). Both diminished rapidly when the dose of cortisone was increased to 200 mg. daily; the basophils increased slightly at first but one week later had almost disappeared, while at the same time the eosinophils had started to return. When the cortisone was stopped, the eosinophils and blood histamine again rose to abnormally high levels. In Case 2, the blood histamine fell in four

[1] Standard deviation.

TABLE II

Histamine and 5-hydroxytryptamine in the blood of healthy children

No.	Age	Sex	Basophils/ cu. mm.	Eosinophils/ cu. mm.	Histamine		Platelets	5-Hydroxytryptamine		
					Blood	Plasma		Buffy coat	Plasma	µg./10^9 platelets
	years				*µg./ml.*		*thousands/ cu. mm.*	*µg./ml. of whole blood*		
1	1½	F					390	0.164	0	0.420
2	2	F	50	97	0.050	<0.003	588	0.165	0	0.280
3	4½	M	50	112	0.045	0.013	362	0.245	0	0.640
4	5	M	73	454	0.080	<0.005	382			
5	6	M	21	557	0.020	<0.003	317			
6	7	M	29	279	0.055	<0.005	272	0.100	0.010	0.404
7	8	M	77	126	0.055	<0.005	167	0.100	0.003	0.370
8	9	M	65	502	0.065	<0.010	324			
9	9	M	64	191	0.055	<0.006	250	0.043	0.048	0.350
10	9	M	22	294	0.023	<0.008	201	0.018	0.098	0.480
11	9	F	56	87	0.068	<0.014	190	0.096	0.045	0.710
12	10	M	34	47	0.050	0.012	197			
13	10	M	28	169	0.043		184			
14	10½	M	64	664	0.075	<0.005	305	0.050	0	0.163
15	11	M	16	81	0.020	<0.011	130	0.016	0.040	0.430
16	12	M	27	183	0.025	<0.006	300	0.125	0	0.410
Mean			45.1	256.2	0.049		266	0.124		0.423
S. D.			20.8	196.7	0.020		111	0.056		0.157

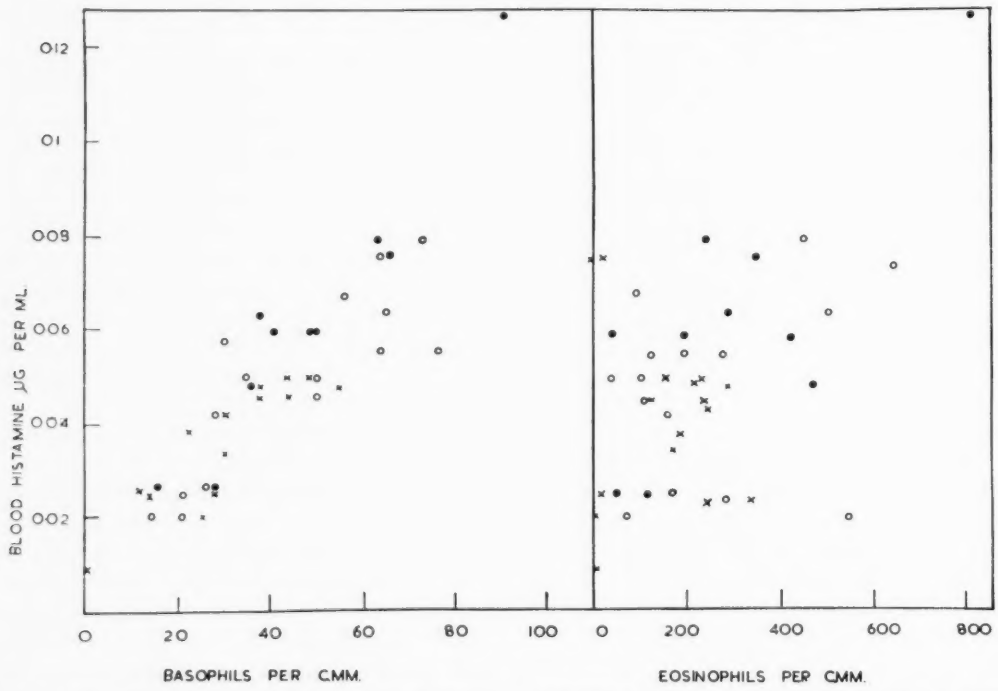

FIG. 1. RELATIONSHIP BETWEEN THE AMOUNT OF HISTAMINE AND THE NUMBERS OF BASOPHILS (LEFT PANEL) AND THE NUMBERS OF EOSINOPHILS (RIGHT PANEL) IN THE BLOOD OF INFANTS AND CHILDREN

Closed circles represent newborn infants; open circles, healthy children; crosses, children with acute rheumatism (Cases 5 and 6).

TABLE III

Histamine and 5-hydroxytryptamine in the blood of children with eosinophilia (Cases 1 to 4) and with acute rheumatism (Cases 5 and 6)

Case	Date	Total leukocytes	Basophils/ cu. mm.	Eosinophils/ cu. mm.	Histamine		5-Hydroxytryptamine	Oral cortisone acetate
					Blood	Plasma		
		thousands/ cu. mm.			*μg./ml.*		*μg./10⁹ platelets*	
1	1/10/57	13.4	66	3,769	0.129		0.338	nil
	1/22/57	13.3	44	4,144	0.146		0.127	nil
	1/24/57	16.7	78	2,991	0.182		0.136	100 mg. daily
	1/28/57	13.3	81	174	0.080		0.142	200 mg. daily
	2/4/57	13.1	6	538	0.055	<0.005	0.250	200 mg. daily
	2/28/57	14.2	41	1,489	0.114	<0.005		nil
2	3/8/57	8.8	59	1,412	0.346	<0.005	0.090	nil
	3/12/57	7.9	63	760	0.110		0.390	nil
	4/18/57	8.2	35	691	0.041		0.060	nil
3	6/4/58	10.4	153	2,335	0.233			nil
	7/2/58	8.3	141	3,627	0.133	<0.007	0.340	nil
	7/30/58	19.4	332	10,260	0.450	<0.003		nil
	8/20/58	13.2	264	8,260	0.311	<0.009	0.194	200 mg. 6 hours previously
4	8/11/58	11.2	113	2,310	0.133	<0.010	0.075	nil
5	5/31/57	9.3	110	17	0.075	<0.010		nil
	6/3/57	16.2	25	2	0.020			200 mg. daily
6	11/19/56	9.1	16	351	0.023		0.368	nil
	11/29/56	8.4	55	295	0.048		0.253	nil
	12/12/56	7.2	28	246	0.023		0.367	nil
	12/20/56	10.9	0	0	0.009		0.272	200 mg. daily
	1/11/57	6.4	13	31	0.025		0.064	75 mg. daily
	1/28/57	11.0	37	128	0.045		0.130	50 mg. daily
	2/15/57	6.8	48	244	0.050		0.025	25 mg. daily
	2/26/57	5.9	30	170	0.033	<0.005	0.080	nil
	3/18/57	5.9	23	191	0.038		0.061	nil
	4/15/57	8.2	30	250	0.043		0.114	nil
	5/16/57	7.7	44	247	0.045		0.127	nil
	12/4/57	6.2	38	224	0.048		0.178	nil
	8/21/58	7.5	44	153	0.050		0.129	nil

days from its initial very high level to just above normal, coincidentally with a fall in the number of eosinophils, while the basophil count remained unchanged. Five weeks later, the basophil count had fallen from 63 to 35 per cu. mm., and the histamine was within normal limits. In Cases 3 and 4, the histamine levels and the basophil and eosinophil counts were abnormally high at every examination. In none of the four cases was histamine detected in the plasma.

Table III also records the results in Cases 5 and 6. In Case 5, eosinophils were virtually absent from the blood on both occasions and the level of blood histamine therefore reflects the basophil count almost directly, since no histamine was found in the plasma. The values obtained in Case 6 show the same relationships as are found in health (Figure 1).

5-Hydroxytryptamine

Ten samples of blood from the umbilical vein were tested for HT; the mean content was 0.012 ± 0.012 μg. per ml. Three of the 10 samples contained no measurable HT and the highest value recorded was 0.027 μg. per ml. Expressed in terms of the platelet count, the mean content was 0.053 ± 0.047 μg. per 10^9 platelets, with a range from 0 to 0.129 μg. per 10^9 platelets (Table I).

HT was measured in the blood of 11 healthy children (Table II). The mean content was 0.124 ± 0.056 μg. per ml. (range, 0.016 to 0.245 μg. per ml.) or 0.423 ± 0.157 μg. per 10^9 platelets (range, 0.163 to 0.71 μg. per 10^9 platelets). The HT content of the blood was thus significantly lower at birth than in later childhood ($p < 0.001$).

The mean value obtained in the eight infants

TABLE IV

5-Hydroxytryptamine in the blood of healthy infants

No.	Age in weeks	Sex	Platelets	5-Hydroxytryptamine		
				Buffy coat	Plasma	μg./10⁹ platelets
			thousands/ cu. mm.	*μg./ml. of whole blood*		
1	7	F	309	0.014	0	0.110
2	7	F	574	0.050	0	0.087
3	10	F	316	0.030	0	0.096
4	13	F	430	0.048	0	0.110
5	15	M	512	0.350	0	0.675
6	19	F	359	0.019	0	0.053
7	20	F	490	0.163	0	0.330
8	29	F	551	0.070	0.04	0.200
Mean			442	0.098		0.197
S. D.			105	0.114		0.212

(Table IV) was 0.098 ± 0.114 μg. per ml. (range, 0.014 to 0.35 μg. per ml.), or 0.197 ± 0.212 μg. per 10^9 platelets (range, 0.053 to 0.675 μg. per 10^9 platelets). The data suggest that an increase in the HT content of the blood occurs rather abruptly at about 16 to 20 weeks of age (Figure 2).

Appreciable amounts of HT were found in the plasma of only seven of the 29 samples, and were attributed to faulty technique, the total blood level (*i.e.*, the content of platelets and plasma) being within normal limits. In about half of the samples, the plasma contained another substance which caused contraction of the uterus, but this was unaffected by lysergic acid diethylamide, and may have been substance P. It was noted that this substance occurred more often in plasma obtained after a meal.

The results obtained in the children with eosinophilia are shown in Table III. The values are very variable, about half being below the lowest value found in the healthy children, and none being abnormally high. Some, but not all, of the low values obtained may have been due to the administration of cortisone. In Case 2, the second value (0.39 μg. per 10^9 platelets) was much higher than the first and third (0.09 and 0.06 μg. per 10^9 platelets, respectively). The total amount of HT in the blood at the second estimation was 0.094 μg. per ml., and since 0.09 μg. of this was in the plasma, it may have represented a truly abnormal plasma level, possibly associated with the allergic condition of the child.

The values obtained from the child with acute rheumatism (Case 6) are also shown in Table III. The HT content of the blood was within the normal range until treatment with cortisone was started when it fell to very low levels. After cortisone was stopped, the HT increased slightly but never regained pretreatment levels.

In the incubation experiments, the platelets from the first newborn infant took up no HT at all, while those from the second, which initially had a total content of 0.01 μg., contained only 0.0375 μg. after incubation. In both the adults, the platelets increased their content nearly sevenfold during incubation.

DISCUSSION

Histamine

With increasing refinements of technique, successive workers have found less and less histamine in plasma, and the recent work of Adam, Hardwick and Spencer (5) indicates that human plasma normally contains less than 1 nanogram (0.001 μg.) per ml. Our method is not adequate for the precise measurement of such amounts, but it would detect an abnormal increase in the plasma histamine. In none of six samples of cord blood plasma was any histamine found and we cannot therefore

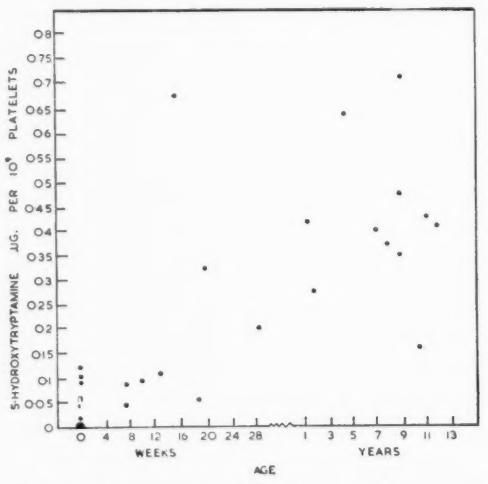

FIG. 2. RELATIONSHIP BETWEEN THE AMOUNT OF 5-HYDROXYTRYPTAMINE IN THE BLOOD OF INFANTS AND CHILDREN AND THEIR AGE IN WEEKS OR YEARS AFTER BIRTH

The increase in the amount of 5-hydroxytryptamine with increasing age is statistically significant ($p < 0.01$).

confirm Pettay's finding of high levels of histamine in the plasma at birth (6). Such high levels may have been due to his method of collecting the blood directly from the placental end of the divided cord.

All except one of the values obtained for the histamine content of cord blood fell within the normal adult range. The one exception (0.130 μg. per ml.) was associated with very high counts of basophils and eosinophils. The mean value (0.063 μg. per ml.) was significantly higher ($p < 0.001$) than the mean of 0.040 μg. per ml. found previously in healthy adults (1) but did not differ significantly ($p > 0.1$) from the mean value of 0.049 μg. per ml. found in the 15 healthy children in this study.

The mean numbers of basophils and eosinophils were rather higher than those recorded previously in umbilical venous blood (15) but the difference is only statistically significant in respect of the eosinophils ($p < 0.05$) and the number of these cells in the blood at birth is known to be extremely variable (18). Figure 1 shows the close relationship between basophils and histamine in the 10 samples.

We conclude that the blood in the umbilical vein does not differ fundamentally from adult blood in respect to the distribution of histamine among the various elements of the blood, and that the tendency to higher levels simply reflects differences in the numbers of circulating basophils and eosinophils.

All the values for blood histamine in the 15 healthy children fell within the normal adult range and the mean value of 0.049 μg. per ml. did not differ significantly ($p > 0.05$) from the mean value of 0.040 μg. per ml. found in adults. Our results are similar to those of Maas, Fehmers and Strengers (4) who estimated the blood histamine in 51 normal children; the mean value calculated from their data was also 0.049 μg. per ml.

The basophil and eosinophil counts in the 15 children all fell within the normal range and the mean counts did not differ significantly from those found in 67 other healthy children (16). Figure 1 indicates that there is a close relationship between the number of basophils and the histamine content of the blood.

Histamine was found in the plasma of only two of the children and the small amounts present were probably due to liberation of histamine from cells into the plasma at the time of collection rather than to true increases in the plasma level. Thus our results indicate that there is no difference between children and adults in the amount and distribution of histamine in the blood.

In general, when the basophil count is high the eosinophil count is likely to be high, and when basophils are scanty, eosinophils are also few in number. Many exceptions to this generalization occur, however, and in a study of 82 adults, Boseila and Uhrbrand (19) found that the correlation between the numbers of basophils and eosinophils was not statistically significant. Nevertheless, in many hundreds of counts we have seldom found a numerical disparity sufficiently great to be of value in defining the role of each type of cell in the carriage of histamine. The observations on the patients with eosinophilia are therefore particularly valuable, since the abnormally high levels of histamine in the presence of normal basophil counts indicate that the eosinophils contained large amounts of the amine. It might be suggested that the basophils were assuming a much greater load than usual, although there is evidence that the histamine content of basophils does not vary much (3). The observations in Case 1, however, show clearly that the eosinophils may at times contain substantial quantities of histamine. Thus, when basophils were virtually absent from the blood on February 4th, the histamine content was 0.055 μg. per ml., and since none of this was in the plasma, it must have been in the eosinophils, which were present in relatively large numbers (538 per cu. mm.). This does not mean, of course, that the eosinophils always contain histamine, and in fact there is ample evidence that they do not. All our children with eosinophilia had abnormally large amounts of histamine in the blood, but this is by no means always the case (20), and the varying quantities found by other workers indicate that the histamine content of the eosinophils may fluctuate greatly, even allowing for possible variations in the numbers of basophils.

The data obtained in Case 5 indicate just as clearly that the basophils contained histamine, since in the presence of large numbers of basophils and in the virtual absence of eosinophils, there was a considerable amount of histamine

TABLE V

5-Hydroxytryptamine in the blood of healthy adults

No.	Sex	Platelets	5-Hydroxytryptamine		
			Buffy coat	Plasma	$\mu g./10^9$ platelets
		thousands/ cu. mm.	*µg./ml. of whole blood*		
1	M	214	0.045	0.011	0.260
2	M	180	0.083	0	0.460
3	F	220	0.094	0	0.427
4	M	180	0.027	0.021	0.150
5	M	235	0.081	0	0.344
6	M	356	0.092	0	0.260
Mean		231	0.076		0.317
S. D.		65	0.022		0.115

which was not in the plasma and must therefore have been in the basophils. The close correlation between the basophils and the blood histamine level in the absence of eosinophils lends further support to the view that the histamine content of each basophil remains relatively constant. The high basophil count in Case 5 is unusual in the acute stage of rheumatism (16) but it may have been due to a poor response to stress in this severely ill child. Basophils are generally less responsive than eosinophils to the action of corticosteroids (3) and may even increase in number at first while the number of eosinophils is falling (21), an effect seen in Case 1 (Table III).

The basophil and eosinophil counts show characteristic changes in acute diseases of childhood (16) but the variations in blood histamine levels have not been studied. However, the data in Cases 5 and 6 do suggest that the same relationships between basophils, eosinophils and blood histamine pertain in acute disease as in health (Figure 1 and Table III). Much work has been carried out on the variations of blood histamine in allergic conditions, and recently it has been shown that some allergic children may have consistently elevated levels of histamine in the blood (4). Such work would be of greater value if the distribution of the histamine between the various blood elements were known. An increase in total blood histamine due to a high level in the plasma, such as occurs in experimental allergic reactions (22, 23), has a significance quite different from that of an increase due to a rise in the numbers of basophils or eosinophils. The latter may, of course, indicate disordered histamine metabolism, since the cells may be numerous because there are increased quantities of histamine to be transported, but equally the blood histamine may only be raised because the numbers of basophils and eosinophils have increased, which may be for some reason unconnected with histamine metabolism. It is evident that in assessing the significance of the amount of histamine in the blood, its distribution between cells and plasma must be taken into account.

5-Hydroxytryptamine

Our results indicate that the blood at birth contains little or no HT, that there is only a slight tendency to rise during the next four months, and that there is then rather a rapid rise to childhood levels, which are comparable with those which we have found in adults (Table V). The rise in the HT content of the blood does not depend on alterations in the platelet count, and is thus due to a real increase in the amount of HT per platelet.

There appear to be at least three possible explanations of the low levels observed at birth. First, the platelets might have been temporarily depleted of HT as a result of parturition, but this seems unlikely, since the rise in the level did not occur for several months after birth. Second, there might have been no HT for the platelets to take up. The apparent absence of 5-hydroxyindole acetic acid from human amniotic fluid (24) seems to support this hypothesis, since it suggests that no HT is being metabolized during fetal life, at least by the normal route. However, we have found that an extract from the terminal ileum of a stillborn fetus caused a contraction of the atropinized estrous uterus equivalent to 2.5 µg. per Gm. of HT, which was abolished by lysergic acid diethylamide. This suggests that HT is present in the human body during intrauterine life, as has already been shown for other mammals (25).

The third, and in our opinion the most likely, explanation of the low levels of HT in cord blood is that the capacity of the platelets for taking up HT is poorly developed at birth. The results of our experiments would appear to support this view, since even after incubation with HT, the platelets of the newborn infants contained less than normal adult platelets.

Our studies afford no explanation for the in-

crease in the platelet content of HT at about four months after birth. It seems unlikely to be related to a change in diet, for the introduction of cereal foods at this age does not greatly increase the intake of tryptophan, and we can only suggest that in some way the ability of the platelets to take up HT matures at about this time.

Histamine and 5-hydroxytryptamine

Although histamine and 5-hydroxytryptamine are closely associated in the cells of some species, such as the mast cells of the rat and the platelets of the rabbit, it appears that in man they are neither derived from the same source nor contained in the same cells. We were therefore surprised to find a significant ($p < 0.01$) inverse relationship between the total quantities of histamine and HT in the blood of the 10 healthy children in whom both substances were measured (Table II). This relationship did not depend on a negative correlation between the basophils and eosinophils and the platelets, and indeed, as far as we are aware, there is normally no numerical relationship between these cells. Under experimental conditions, HT does have an eosinopenic effect (*26, 27*) and may also liberate histamine (28), but the quantities required to produce these effects are much larger than those found in normal blood, where in any case the HT is within the platelets and therefore unlikely to exert any such action.

We cannot confirm this relationship from our other results, which are not comparable because of the difference noted in the uptake of HT by platelets in the newborn period and because any negative correlation would cease to exist on the administration of cortisone, which lowers the levels of both histamine and HT (1, 29). If there is indeed an inverse relationship between histamine and HT in human blood, we are unable to account for it.

SUMMARY

1. The amount of histamine in the blood is closely related to the number of circulating basophils at birth and throughout childhood, and there is little or no histamine in the plasma. Fresh evidence that both basophils and eosinophils contain histamine has been obtained from observations on children with eosinophilia and with acute rheumatism.

2. 5-Hydroxytryptamine in the blood in infancy and childhood is almost entirely contained in the platelets and little or none is present in the plasma. There is very little 5-hydroxytryptamine in the blood at birth but the amount increases rapidly at about the age of four months, and thereafter remains throughout childhood at levels comparable with those found in adult life.

3. The increase at four months is due to an increase in the amount of 5-hydroxytryptamine per platelet and not to an increase in the number of platelets. Experimental evidence suggests that the platelets contain only a small amount of 5-hydroxytryptamine at birth because their capacity for taking it up is poorly developed.

4. The values for 5-hydroxytryptamine in the blood of patients with eosinophilia were variable and observations on one patient indicated that abnormally large amounts might on occasion be present in the plasma.

5. An inverse relationship was found between the amounts of histamine and 5-hydroxytryptamine in the blood of healthy children. The physiological significance of this observation is not known.

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THE EFFECT OF HIGH PERFUSION PRESSURES ON THE GRANULATION OF JUXTAGLOMERULAR CELLS IN AN ISOLATED KIDNEY *

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(Submitted for publication October 10, 1958; accepted November 25, 1958)

In our previous studies on the granulation of juxtaglomerular cells, we were impressed with several experiments in which a kidney was perfused with blood at a high pressure and had a striking decrease in the juxtaglomerular granulation (1). Conversely, in other situations where the pressure of the blood perfusing the kidney was reduced, the juxtaglomerular granulation tended to increase (1). These relationships can easily be indirect or coincidental, and it seemed of interest to find out whether changes in perfusion pressure can directly alter the granulation of these cells.

METHODS

In this experiment, we would have preferred to test perfusion pressures both higher and lower than normal. However, when a clip is allowed partially to constrict one renal artery in a rat for 24 hours, no increase in juxtaglomerular granulation occurs in the clipped kidney at the end of the 24 hours, even though such an increase usually occurs after a few weeks. This experiment suggested that lowering the perfusion pressure would not produce any detectable changes within the space of a four hour perfusion experiment. Hence, we were prompted to compare perfusion pressures slightly below normal with distinctly high perfusion pressures. The design of our perfusion apparatus is illustrated diagrammatically in Figure 1. A normal male Wistar rat (A) provided oxygenated and "biochemically supported" blood through its carotid artery (B) to some Tygon tubing (C) which ran through a Sigmamotor® pump (D). The pump could be regulated to increase or decrease the pressure of blood flowing into the Tygon tubing distal to it, which was connected to the renal artery of an isolated normal rat kidney (E). The isolated kidney was always obtained from a normal male Wistar rat and was removed in such a way that it never had more than 15 seconds of ischemia during the "hooking-up" period before being placed in the warm chamber (F). This warm chamber contained a space which just fitted the kidney and served to maintain it at body temperature. The isolated kidney was wrapped in a Saran® plastic film to reduce evaporation and to prevent any contact with the potentially toxic heavy metals of the warm chamber. A side arm in the tubing between the pump and the kidney was connected to a mercury manometer (G) which was monitored continuously to determine whether the pump required any adjustment to maintain a given constant perfusion pressure. The blood leaving the isolated kidney via the renal vein went through polyethylene tubing to a miniature Murphy drip apparatus (H), which provided an estimate of the magnitude of flow. From there, it returned through some more polyethylene tubing to the jugular vein of the original rat (I).

Heparin was added to the circulating blood to prevent clotting. The "blood donor" rat (A) was anesthetized with Nembutal®. His trachea was cannulated to prevent any airway obstruction. The perfusions lasted from one to four hours. Sixteen kidneys were perfused at about 85 mm. Hg, a level on the low side of normal but one at which renal autoregulation is considered to be intact (2). These perfusions at a relatively low pressure were compared with 21 other experiments where the perfusion pressure varied between 167 and 192 mm. Hg.

When the isolated kidney intended for perfusion was first obtained, the opposite kidney was also removed and subsequently sectioned. The juxtaglomerular indices in both kidneys were estimated using the Hartroft method (3) as employed in our laboratory (1). Six different sections from each kidney were counted and then averaged, thereby increasing our precision two and one-half

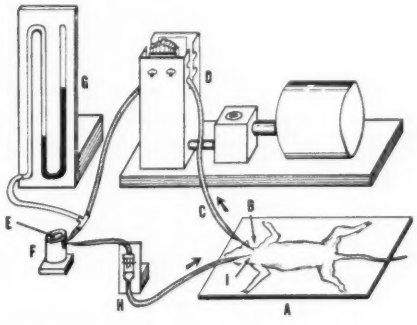

FIG. 1. DIAGRAM OF APPARATUS USED IN PERFUSING AN ISOLATED RAT KIDNEY

* This investigation was supported by a grant from the American Heart Association and by Grant No. H 2008 from the National Heart Institute, United States Public Health Service.

times over that which was obtained when counting single sections. It was assumed that the two kidneys would have approximately the same juxtaglomerular index. This assumption was tested (see under Results below). By determining the juxtaglomerular index of the isolated kidney at the end of the perfusion and by comparing it to the juxtaglomerular index of the control opposite kidney from the same rat, one can get a good estimate of the changes in juxtaglomerular granulation that occurred during the perfusion.

Since the peristaltic action of the Sigmamotor® pump is more active during high pressure perfusions, more hemolysis would occur. To control this factor, some cross-matched homologous rat blood was homogenized, and given portions were injected into the circulation every 10 minutes during some of the low pressure perfusions. This provided the same degree of hemolysis that was found at the termination of the "high pressure" perfusions.

We were curious to know whether there was some humoral factor being introduced into the renal vein of the isolated kidney that was influencing the granulation of the juxtaglomerular cells. To investigate this possibility, the juxtaglomerular index was determined in a number of kidneys of "blood donor" rats ("A" in Figure 1), as well as in the isolated kidney.

To determine whether a change in adrenal secretion had any effect on the experiment, a few "blood donor" rats were totally adrenalectomized just before beginning the experiment. These adrenalectomized rats were given one injection of 3 mg. of cortisone acetate subcutaneously just after the adrenalectomy. A few such adrenalectomized rats were used in high as well as "low pressure" perfusions. Any given rat might be used in either a "high pressure" or a "low pressure" perfusion experiment but never in both.

The blood flow in the isolated kidneys varied between 0.2 and 4.0 ml. per minute. The resistance in the renal vascular bed was estimated by the following equation:

$$\text{Resistance} = \frac{\text{Renal arterial pressure (mm. Hg)-renal venous pressure (mm. Hg)}}{\text{renal blood flow (ml. per min.)}}.$$

The resistance was measured in so-called "PRU_m" resistance units (mm. Hg per ml. per minute) (4). The resistance in the isolated kidneys varied between 60 and 500 units. At the beginning of each experiment, the perfusion pressure was kept around 85 to 95 mm. Hg and the resistance in the isolated kidneys averaged 224 units and ranged between 100 and 406 units. If the perfusion pressure was maintained at this relatively low level, the resistance in the renal vascular bed usually dropped to around 100 units after 20 minutes, with a concomitant increase in blood flow. The resistance in these kidneys would then gradually increase about 50 units after one and one-half hours of perfusion. Figure 2 illustrates a typical perfusion at lower pressure.

When the kidneys were perfused at the higher pressures (167 to 192 mm. Hg), half of them would show an attempt at "autoregulation" with the resistance in the renal vascular bed rising 200 to 300 units shortly after the higher perfusion pressure was applied. Eventually, all the kidneys perfused at the higher pressures had a drop in resistance to levels between 50 and 100 resistance units. After 30 to 80 minutes in this state of lowered vascular resistance, the renal resistance would gradually begin to rise and would continue up to levels of 350 to 500 units. At this point the blood flow in the isolated kidneys was down to the starting level and the perfusions were terminated. A typical "high pressure" experiment is illustrated in Figure 3.

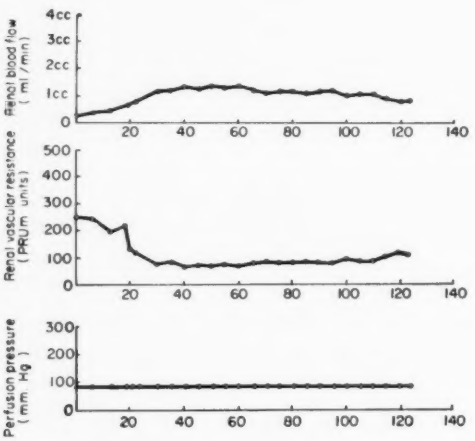

FIG. 2. PERFUSION PRESSURE, RENAL BLOOD FLOW, AND RENAL VASCULAR RESISTANCE IN A TYPICAL "LOW PRESSURE" PERFUSION EXPERIMENT

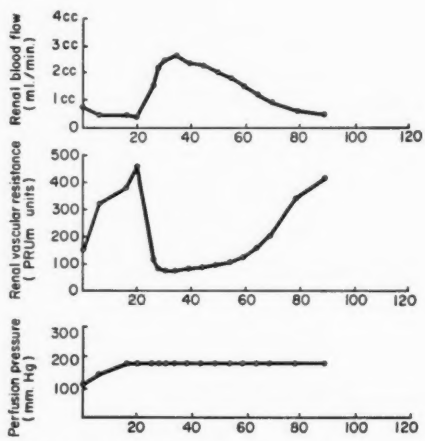

FIG. 3. PERFUSION PRESSURE, RENAL BLOOD FLOW, AND RENAL VASCULAR RESISTANCE IN A TYPICAL "HIGH PRESSURE" PERFUSION EXPERIMENT

TABLE I

Perfusion experiments using high pressures

Experiment*	Perfusion pressure	Time	JG index of control kidney (JG_c)	JG index of isolated perfused kidney (JG_p)	"% of change" $\left(\frac{JG_p}{JG_c} \times 100\right)$	JG index of kidney of "blood donor" rat
	mm. Hg	*min.*				
A	178	130	35	7	20	
B	167	175	34	9	26	
C†	182	130	27	10	37	32
D	188	70	41	16	39	
E	182	77	51	21	41	34
F	182	90	45	19	42	
G	192	128	36	17	47	
H	173	100	48	23	48	
I	178	130	48	23	48	
J†	182	85	44	25	57	44
K	182	131	49	28	57	39
L	182	185	42	26	62	49
M	182	240	56	36	64	
N	167	232	43	28	65	
O	173	140	49	34	69	
P†	178	110	58	42	72	42
Q	188	115	44	32	73	
R	173	140	51	40	78	
S	182	85	45	37	82	33
T	192	60	41	35	85	
U	188	75	40	44	110	
Mean			44	26	58	39
Standard deviation			±7.41	±10.86	±21	±6.38

* Listed in the order of increasing "per cent of change."
† The "blood donor" rat was adrenalectomized before commencing the experiment.

There was a period of lowered vascular resistance in the middle of all the perfusions. In the perfusions at high pressures the maximum blood flow in the kidneys averaged 2.6 ml. per minute. In the perfusions at 85 to 95 mm. Hg, the maximum flows averaged 1.0 ml. per minute.

RESULTS

Tables I and II give the results for each individual experiment. They give the perfusion pressure, the length of the perfusion, and the juxtaglomerular (JG) indices for the control kidney and the isolated perfused kidney as well as the "per cent of change" between the two. In some instances, they give the JG index of a kidney residing in the "blood donor" rat which provided the oxygenated blood. The experiments with perfusions at high pressures are described in Table I. Those at lower pressures are shown in Table II. In the experiments with perfusion at high pressure, there was a highly significant degree of degranulation of the juxtaglomerular cells as a result of the perfusion ($p < 10^{-8}$). The juxtaglomerular index dropped from a mean of 44 in the control kidney to a mean of 26 in the perfused kidney. This is to be contrasted with the perfusions at lower pressures. The control kidneys had a mean juxtaglomerular index of 43, while the kidneys perfused at the lower pressure also had a mean juxtaglomerular index of 43. Thus, perfusion at higher pressures caused significant degranulation, while perfusion at lower pressures did not. Also in Tables I and II, the JG index of each perfused kidney is presented as a given percentage of the JG index of its own control kidney ($JG_p/JG_c \times 100$). Calculating the results in this way cancels out the inherent variation in the JG indices of the control kidneys from rat to rat. The kidneys perfused at the lower pressures averaged 100 per cent of their control values, indicating that they had virtually the same JG indices as their control kidneys. On the other hand, the kidneys perfused at the higher pressures averaged only 58 per cent of their control kidneys. When this difference was analyzed statistically, it proved to be highly significant ($p < 10^{-12}$). Some of the perfusions at the higher pressure continued for little more than an hour. In these relatively

TABLE II

Perfusion experiments using low pressures

Experiment*	Perfusion pressure	Time	JG index of control kidney (JG_c)	JG index of isolated perfused kidney (JG_p)	"% of change" $\left(\frac{JG_p}{JG_c} \times 100\right)$	JG index of kidney of "blood donor" rat
	mm. Hg	*min.*				
a†	82	110	53	46	87	35
b†	82	120	38	33	87	43
c†	82	110	48	43	90	41
d†	82	120	41	37	90	35
e	82	240	44	40	91	
f†	85	85	50	46	92	43
g	85	210	42	40	95	
h	82	240	47	45	96	
i	85	240	48	46	96	
j	82	240	51	51	100	
k†	82	120	41	42	102	40
l	85	240	32	34	106	
m‡	82	120	39	43	110	44
n	100	240	39	44	113	
o‡	85	105	41	47	115	49
p†	85	120	36	45	125	31
Mean			43	43	100	40
Standard deviation			±5.92	±4.88	±11	±5.56

* Listed in the order of increasing "per cent of change."
† Homogenized matched blood was given intravenously to the "blood donor" rat.
‡ The "blood donor" rat was adrenalectomized before commencing the experiment.

short perfusions, a sizable drop in juxtaglomerular granulation did not always occur.

In comparing the perfused kidney with the control kidney, we assumed that the indices of the two kidneys would be quite similar at the start of the experiment. This assumption was tested in five rats (see Table III) and the two kidneys in any given rat never differed by more than 21 per cent.

Since adrenalectomy increases the granulation of the juxtaglomerular cells and desoxycorticosterone decreases it (1, 3, 5), the adrenals obviously affect juxtaglomerular granulation and it seemed important to determine whether an increased rate of secretion by intact adrenals was necessary to get the degranulation during perfusions at high pressure. Five rats were adrenalectomized and given cortisone acetate just before serving as the blood donors in the experiment. Three of the rats were utilized in perfusions at high pressure and significant degranulation occurred in the perfused kidney in each instance (see Table I). The other two adrenalectomized rats were utilized in perfusions at lower pressures (see Table II). In both rats, no degranulation whatsoever took place. These experiments rule out fairly well the possibility that an augmented secretion from the adrenals is responsible for the juxtaglomerular degranulation in the isolated kidney.

TABLE III

Juxtaglomerular granulation of the right and left kidneys of normal rats

Rat no.	Juxtaglomerular index of right kidney	Juxtaglomerular index of left kidney
1	38	39
2	38	40
3	40	34
4	35	43
5	50	52
Mean	40	41

The possibility also arose that perfusion at high pressure causes the isolated kidney to release a humoral substance into the blood leaving its renal vein. Such a substance could directly or indirectly cause the reduction of juxtaglomerular granulation in the isolated kidney. Such a humoral substance would also be expected to produce a degranulation of the juxtaglomerular cells of the kidneys residing within the "blood donor" rat which is providing the oxygenated blood. However, the average index of such kidneys from seven experiments utilizing perfusion at high pressure was 39, while the average index of

such kidneys from nine experiments with perfusions at lower pressures was 40 (Tables I and II). Since this difference is negligible, the possibility that a humoral substance in the general circulation produces the degranulation at high pressures is remote.

Varying degrees of hemolysis occurred during the perfusion experiments. In general, there was more hemolysis during the perfusions at higher pressure. However, some experiments at the higher pressures had very little hemolysis and the typical degranulation of the juxtaglomerular cells still occurred. Moreover, given portions of matched hemolyzed blood were injected into the general circulation every 10 minutes in seven of the "low pressure" perfusion experiments. This introduction of hemolyzed blood provided more hemolysis than was seen in any of the experiments with perfusions at high pressure and still no significant degranulation occurred in the juxtaglomerular cells of the isolated kidney (Table II). It would appear that the juxtaglomerular degranulation in the perfusions at high pressure is not related to hemolysis.

DISCUSSION

The data here strongly support the hypothesis that an increase in perfusion pressure brings about a degranulation of the juxtaglomerular cells. Since the higher perfusion pressures were associated on the average with higher renal blood flows, the degranulation could be primarily related to flow rather than pressure. A number of rats had maximum renal flows of less than 2 ml. per minute during perfusions at high pressure and still had significant degranulation. On the other hand, one rat had a renal flow of about 2 ml. per minute with a perfusion pressure of 85 mm. Hg. There was no significant change in juxtaglomerular granulation in this instance. Nevertheless, these kidneys might have had quite a different flow at higher or lower perfusion pressures. Hence, one cannot say with complete assurance that the degranulation is strictly dependent on the higher pressure. A higher renal blood flow or a higher rate of glomerular filtration, either of which could have resulted from a higher perfusion pressure, could be partly responsible for the degranulation.

However, the observations are quite compatible with the theory that the juxtaglomerular cells residing within the walls of the afferent glomerular arterioles can act as stretch receptors, changing their rate of secretion as the arteriolar wall changes its degree of stretch. Varying the perfusion pressure would be expected to produce just such an alteration in the stretch of the afferent arterioles.

In the mammalian circulation, the pressure in the arterial system mechanically stimulates baroreceptors in the carotid sinus, in the aortic arch, and in or near the arteries supplying the pancreas and intestines (6). It would seem that the juxtaglomerular cells constitute still another possible receptor area for the appreciation of changes in blood pressure. Such a theory need not imply that mechanical factors are the only stimuli for the juxtaglomerular cells. It is obviously quite possible that the concentration of mineralo-corticoids or of sodium ions in the extracellular fluid could also be important in determining the rate of secretion of the JG cells. The possible functions of these cells have been discussed previously (1).

SUMMARY

Twenty-one isolated normal kidneys were perfused with oxygenated, "biochemically supported" blood at pressures ranging between 167 and 192 mm. Hg. The degree of granulation of the renal juxtaglomerular cells (the juxtaglomerular index) dropped on the average from 44 to 26 during the one to four hour perfusion at these high pressures. Sixteen similar isolated kidneys were perfused at pressures ranging between 82 and 85 mm. Hg. In these kidneys, the average JG index was 43 at the start of the perfusion and still remained 43 at the end of the perfusion. The difference between the two groups was highly significant ($p < 10^{-12}$). Thus, high perfusion pressures produce marked degranulation of the juxtaglomerular cells in a short period of time, while perfusion at fairly normal pressures produces no change in juxtaglomerular granulation.

Supplementary experiments indicate that the degranulation of juxtaglomerular cells at high perfusion pressures is not caused by an augmented secretion of the adrenal, is not caused by

the release of humoral substances into the renal vein, and is not related to the degree of hemolysis in the experiment.

These observations are compatible with the theory that the juxtaglomerular cells can act as baro-receptors, changing their rate of secretion as the level of arterial pressure changes.

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ANNOUNCEMENTS OF MEETINGS

The Fifty-First Annual Meeting of THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in Atlantic City, N. J., on Monday, May 4, 1959, at 9:00 A.M. at the Casino Theater on the Steel Pier.

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IN VITRO STUDIES WITH 16-C^{14}-ESTRONE: DISTRIBUTION BETWEEN PLASMA AND RED BLOOD CELLS OF MAN *

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(Submitted for publication October 10, 1958; accepted December 4, 1958)

Albrieux has reported that the blood cells of nonpregnant women contained at least twice as much estrogenic activity as the blood serum, while during pregnancy the estrogenic activity was equally distributed between cells and serum (1, 2); however, the specificity of the assay used in these studies is questionable.

Bischoff, Katherman and Yee, using the uterine weight of immature rats as bioassay technique, investigated the *in vitro* distribution of estrogens in blood (3, 4); after an equilibrium period of 10 minutes at 37.5° C., it was found that the ratio of biological activity in red cells to that in serum of rabbit blood was from 0.39 to 0.11 for estrone. An increase in the total recovery of biologic activity could be produced by prolonging incubation, thus suggesting a partial transformation of estrone into a more biologically active steroid (4); the conditions of this activation were studied in detail (5) and Gray and Bischoff demonstrated quite conclusively that the increase in activity was due to the conversion of estrone to estradiol by human and rabbit red cells (6).

Sandberg, Slaunwhite and Antoniades have observed that approximately 30 per cent of the 16-C^{14}-estrone added to a sample of whole blood remained with the red cells, while in a reverse dialysis experiment 55 per cent of the radioestrone was associated with the red cells (7).

The present work is an attempt to study further the *in vitro* distribution of 16-C^{14}-estrone between plasma and red blood cells of man.

METHODS

Experimental subjects. The heparinized blood samples used in the present study were drawn from normal adult individuals. Subjects B, C and E were females, 24, 31 and 28 years of age, respectively, while Subjects A, D and F were males, 28, 33 and 32 years of age, respectively.

Centrifugation of the samples. An International Centrifuge, size 2, model V, with a No. 240 head and 100 ml. cups giving a rotating radius of 25 cm. at the tip of the cups was used. All samples were centrifuged at 2,000 rpm for 30 minutes; the relative centrifugal force was approximately 1,100 times gravity at the tip.

Microhematocrits. Duplicate hematocrits were run on each blood sample using an International Micro-capillary Centrifuge (Model 490) and heparinized capillary tubes of 75 mm. in length and 1.0 mm. in diameter. The tubes were centrifuged at 11,000 rpm for five minutes and the percentage of blood cell volume was read on a microcapillary reader (8).

Purity and standardization of 16-C^{14}-estrone. The 16-C^{14}-estrone was purchased from Charles E. Frosst Co., Montreal, Canada. Its specific activity was 0.729 mc. per mM. The purity of this material was tested as previously described (9); 92 to 95 per cent of the radioactivity of an aliquot chromatographed on paper was recovered in the area of the chromatogram corresponding to an estrone standard.

An ethanolic solution of the steroid was prepared in such a manner that it contained 50,300 cpm per ml.

Mode of extraction of the various specimens. Plasma samples as well as all the other aqueous samples (saline solution, Krebs-Ringer solution) were extracted five times with twice their volume of redistilled chloroform. The chloroform extracts were evaporated to dryness.

The red blood cells were kept overnight in the deep-freeze and then thawed. Three times their volume of 100 per cent ethanol was added and mixed rapidly. The mixture was centrifuged and the supernatants were decanted. The residue was washed with ethanol (equal in amount to the initial red cell volume) and after centrifugation the supernatants were added to those previously prepared and the resultant specimens were subsequently evaporated to dryness.

Paper chromatography and radioautography. Some of the extracts were applied on Whatman No. 2 paper and chromatographed with the system of solvents isooctane: toluene: methanol: water (25: 75: 80: 20) described by De Courcy (10). The R_f values of various estrogens in this system have been reported elsewhere (9). The chromatograms were then radioautographed as previously described (9).

Radioactivity determination. The dry residue of the various extracts was dissolved in a known volume of ethanol and an aliquot was assayed for radioactivity as previously described (11). The assays were made in a

* This work was made possible by a grant-in-aid from the National Institutes of Health, United States Public Health Service.

TABLE I

In vitro distribution of 16-C^{14}-estrone between the plasma and the red cells of various blood samples

Subjects*	Sample*	Blood vol.	Blood hemat.	Expected plasma vol.† + 1.3 ml.‡	Obtained plasma vol.	Per cent activity	
						In obtained plasma vol.	Expected in plasma§
		ml.		*ml.*	*ml.*		
A	1	30	38	18.6 + 1.3 = 19.9	18.25	74.8	81.5
	2	30	38	18.6 + 1.3 = 19.9	18.0	76.5	84.5
B	1	30	37.5	18.75 + 1.3 = 20.05	18.25	71.7	78.8
	2	30	37.5	18.75 + 1.3 = 20.05	18.25	68.2	75.0
C	1	20	40	12.0 + 1.3 = 13.3	12.5	77.2	82.2
	2	17	40	10.2 + 1.3 = 11.5	11.0	80.0	83.6
D	1	20	44	11.2 + 1.3 = 12.5	11.7	80.0	85.5
	2	20	44	11.2 + 1.3 = 12.5	11.6	82.0	88.4
	3	20	44	11.2 + 1.3 = 12.5	11.9	78.2	82.2

* In Subjects A and B, the blood samples were allowed to stand 30 minutes at room temperature prior to centrifugation, while in Subjects C and D the samples were centrifuged immediately (Experiment No. 1).

† $\left(\text{Blood volume} \times \frac{100 - \text{hematocrit}}{100}\right)$.

‡ Volume of radioestrone solution plus volume of saline solution.

§ Per cent activity expected in plasma equals per cent activity in obtained plasma vol. $\times \frac{(\text{exp. plasma vol.} + 1.3)}{(\text{obtained plasma vol.})}$.

"micromil" window gas flow counter (model D-47, Nuclear, Chicago).

EXPERIMENTS AND RESULTS

Experiment No. 1

A series of 50 ml. centrifuge tubes were prepared containing 0.3 ml. of the standard solution of 16-C^{14}-estrone, 1 ml. of 0.9 per cent NaCl solution and various amounts of blood drawn from Subjects A, B, C and D. The tubes of Subjects C and D were centrifuged immediately while the tubes of Subjects A and B were allowed to stand for 30 minutes at room temperature (with occasional gentle mixing) prior to centrifugation. Plasma was removed quantitatively from each tube using a capillary pipette and measured carefully. The volume of plasma obtained was compared with the plasma volume expected on the basis of blood microhematocrit, taking into consideration the volumes of the radioestrone solution and saline solution added to each tube (see Table I).

Each plasma sample was extracted with chloroform. The extracts were evaporated to dryness and the residue was assayed for its radioactivity. The results were corrected on the basis of the expected plasma volume. As can be seen from Table I, 75.0 to 88.4 per cent of the radioactivity was calculated to be in the plasma of various blood samples, while the balance of the activity could be expected to accompany the red cells, *i.e.*, 25 to 11.6 per cent. It is to be noted that the results were not significantly different whether the blood samples were immediately centrifuged (Subjects C and D), or whether they were allowed to stand for 30 minutes.

Experiment No. 2

Nine centrifuge tubes were prepared, each one containing 0.2 ml. of radioestrone solution, 1 ml. of saline solution and 10 ml. of blood from Subject E. Three of them were placed in an incubator at 37° C., three were kept at room temperature (22° C.) and the remaining three maintained at 5° C. in a cold room. One tube of each group was centrifuged after standing for 30 minutes, another was allowed to stand for 120 minutes, while the last tube was kept 240 minutes prior to centrifugation. Following centrifugation, the various samples were treated as described in Experiment No. 1.

The results are shown in Table II and it can be seen that neither time nor temperature influenced the distribution of the radioactivity between plasma and red cells.

TABLE II

Effect of temperature and time on the in vitro distribution of 16-C^{14}-estrone between plasma and red cells of blood samples from Subject E (Experiment No. 2)

Experimental conditions						Per cent activity			
Temperature	Time	Blood vol.	Blood hemat.	Expected plasma volume + 1.2 ml.	Obtained plasma volume	In obtained plasma vol.	Expected in plasma	Found in RBC layer	Total recovery
	min.	*ml.*		*ml.*	*ml.*				%
	30	10	39	6.1 + 1.2 = 7.3	6.5	79.0	88.8	15.7	94.7
5° C.	120	10	39	6.1 + 1.2 = 7.3	6.5	78.5	88.2	19.7	98.2
	240	10	39	6.1 + 1.2 = 7.3	6.4	79.6	90.8	18.3	97.9
	30	10	39	6.1 + 1.2 = 7.3	6.5	78.0	87.3	18.5	97.5
22° C.	120	10	39	6.1 + 1.2 = 7.3	6.6	80.4	88.9	19.0	99.4
	240	10	39	6.1 + 1.2 = 7.3	6.4	77.8	88.8	18.2	96.0
	30	10	39	6.1 + 1.2 = 7.3	6.6	81.3	90.0	16.5	97.8
37° C.	120	10	39	6.1 + 1.2 = 7.3	6.4	78.0	88.9	15.7	93.7
	240	10	39	6.1 + 1.2 = 7.3	6.5	78.0	87.6	16.3	94.3

Experiment No. 3

In Experiment No. 1, following removal of plasma, the red cells of blood of the various samples were saved for the present experiment. The red cells of Sample 1 of Subject B and of Sample 2 of Subject C were washed with their volume of 0.9 per cent NaCl solution, while the red cells of Sample 1 of Subject D were washed with twice their volume saline; the red cells of Sample 1 of Subject C and Sample 2 of Subject D were washed with their volume of plasma (the plasma used for the washings came from the corresponding subjects). This procedure was carried out four times for the plasma washings and 10 times for the saline washings. Each of the washings was kept separately and extracted with chloroform. After these repeated washings, the red blood cells were precipitated and extracted with ethanol.

The radioactivity detected in the various extracts is shown in Figure 1, while the total recoveries are reported in Table III. When the logarithms of the washing values were plotted

TABLE III

Distribution coefficient of 16-C^{14}-estrone in the systems red blood cells and 0.9 per cent NaCl solution, or Krebs-Ringer solution, or plasma (Experiment No. 3)

	Washings			Distribution coefficient* Mean ± S.D.	Total recovery (in % of radio-estrone added)
	Type of washings	Volume	Number of washings		
Sample 1, Subj. D	0.9% NaCl	Twice RBC vol.	×10	0.197 ±0.010	104.56
Sample 2, Subj. C	0.9% NaCl	Once RBC vol.	×10	0.110 ±0.012	96.86
Sample 1, Subj. B	0.9% NaCl	Once RBC vol.	×10	0.094 ±0.009	93.56
Sample 1, Subj. C	Plasma	Once RBC vol.	× 4	3.18	93.57
Sample 2, Subj. D	Plasma	Once RBC vol.	× 4	3.25	104.11
Subj. E	Krebs-Ringer	Once RBC vol.	×10	0.098 ±0.008	98.05
Subj. F	Krebs-Ringer	Once RBC vol.	×10	0.099 ±0.011	97.62

* Washing No. 1 excluded.

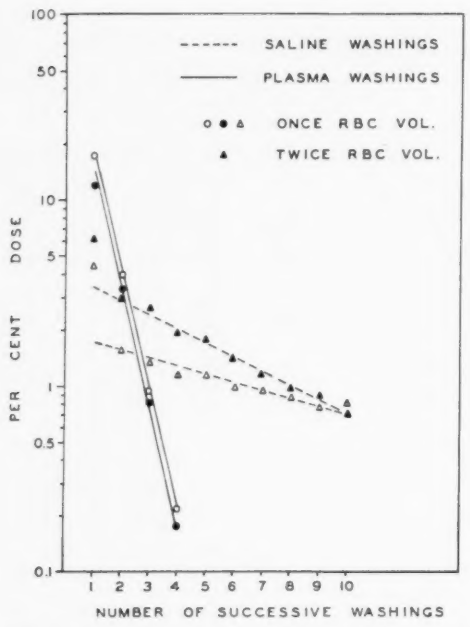

FIG. 1. RADIOACTIVITY IN SUCCESSIVE PLASMA WASHINGS OF TAGGED RBC, COMPARED WITH THAT OBTAINED IN SUCCESSIVE 0.9 PER CENT NaCl WASHINGS (EXPERIMENT No. 3)

(Figure 1) these values fell on a straight line with the exception of Washing No. 1. This is characteristic of the partition of a compound between two immiscible solvents. The distribution coefficient was calculated by two different methods. In the first method, the following formula was used:

$$K = \frac{\text{Wash No. 10}}{\text{RBC}} = \frac{\text{Wash No. 9}}{\text{RBC} + \text{Wash No. 10}}$$

$$= \frac{\text{Wash No. 8}}{\text{RBC} + \text{Wash No. 10} + \text{Wash No. 9}}$$

$$= \cdots \text{and so forth.}$$

In Method 2, the formula employed was:

$$K = \frac{\text{Wash No. 1}}{\text{Wash No. 2}} - 1 = \frac{\text{Wash No. 2}}{\text{Wash No. 3}} - 1$$

$$= \frac{\text{Wash No. 3}}{\text{Wash No. 4}} - 1 = \cdots \text{and so forth.}$$

The results of these calculations are shown in Table III. Except for the distribution coefficients calculated on the basis of the activity in Wash No 1, the K values obtained were relatively constant. However, Method 1 gave a greater consistency than Method 2. This is probably due to the fact that small technical errors are reflected more strongly in the distribution coefficient calculated by the latter method; furthermore, no value could be obtained by this method when the activity in a washing was less than that obtained in the following wash.

In a similar experiment, where Krebs-Ringer solution was used for the washing of the red cells (Subjects E and F) instead of saline solution, identical results were obtained (Table III).

Experiment No. 4

Two centrifuge tubes were prepared containing 0.3 ml. of radioestrone, 9.7 ml. of saline solution and 10 ml. of red blood cells (without white cells) from Subject D. The tubes were shaken gently, but thoroughly, and were allowed to stand at room temperature for 30 minutes with occasional mixing. At the end of this time, the tubes were centrifuged and the saline solution was removed. The red cells were washed nine more times with 10 ml. of saline solution, and at the end of this procedure they were precipitated and extracted with ethanol.

The radioactivity remaining with the red cells represented 33.3 and 37.3 per cent of the estrone added. The values for the various saline washings, with the exception of the first one, fell on a straight line when plotted on semilogarithmic paper. The average distribution coefficients obtained by Method 1 were 0.090 ± 0.011 and 0.091 ± 0.002, while the total recoveries were 93.11 and 96.29 per cent of the radioestrone added.

Experiment No. 5

This was carried out to test the loading capacity of the red cells. The technique was similar to that described in the previous experiment, with the exception that in the first step the washed red cells from Subject D were loaded with nonradioactive estrone (5 to 100 μg.). Table IV summarizes the method and shows the results. It can be seen that the amount of radioactivity with the red cells was independent of the amount of nonradioactive estrone used in the first step of the experiment.

There was some doubt whether or not the total amount of nonradioactive steroid had been completely picked up by the red cells. In order to check on this point, the first step of the experiment was repeated and the saline washings were kept, pooled and extracted with chloroform. The chloroform was evaporated to dryness and a Kober reaction was performed on the residue. A known amount of estrone standard was added to the residue of Tube 6; only 80 per cent of the optical density expected from the amount of estrone standard added was obtained, the inhibition probably being due to the material resulting from slight hemolysis of red cells. Taking this value as standard, it was found that Tubes 1 to 5 contained, respectively, 11.2 13.0, 16.3, 13.6 and 18.0 per cent of the amount of nonradioactive estrone added. It would, therefore, appear that most of the estrone was picked up by the red cells in the first step of the experiment, but these cells were not overloaded since they were able to pick up the radioactive estrone in the second step of the test. In other words, the solubility of estrone in red cells is at least 82 μg. per 5 ml. (in Tube 5) or 6.08×10^{-5} M per L.

Experiment No. 6

A suspension of stroma (Subject E) in 1 : 10,000 acetic acid solution was prepared following the method described by Tischkoff, Robscheit-Robbins and Whipple (12). The stroma was separated from the liquid phase and then washed with saline solution in order to remove any trace of acetic acid solution.

A volume of 2.3 ml. of saline solution and 0.1 ml. of radioactive estrone was added to 2.4 ml. of stroma. After thorough mixing, the tube was centrifuged and the liquid phase was removed. Three more washings with 2.4 ml. of saline solution were carried out in a similar fashion. The washings were extracted with chloroform while the stroma residue was precipitated and extracted with ethanol as described for the red cells. The total recovery was 94.5 per cent and the stroma residue contained 65 per cent of the amount of the radioactivity added at the beginning of the experiment. The average distribution coefficient of estrone in the system stroma : saline solution was calculated to be 0.095 and was similar to that obtained for the system intact red cells : saline solution.

Experiment No. 7

A solution of hemoglobin in saline (Subject D) was prepared as follows: 30 ml. of 0.9 per cent NaCl solution was added to 10 ml. of washed red cells which had been separated from the white blood cells; the mixture was frozen and thawed

TABLE IV

Loading of RBC with nonisotopic estrone followed by tagging with 16-C^{14}-estrone (Experiment No. 5)

			Tube number					
			1	2	3	4	5	6
First step	a. Mix—centrifuge Saline discarded	Nonradioestrone (100 μg./ml.)	0.05 (5 μg.)	0.10 (10 μg.)	0.20 (20 μg.)	0.50 (50 μg.)	1.0 (100 μg.)	0
	b. Add 5 ml. saline Mix—centrifuge Saline discarded	0.9% saline solu. Washed red cells	9.95 5.0	9.90 5.0	9.80 5.0	9.50 5.0	9.0 5.0	10.0 5.0
Second step	a. Mix—centrifuge Saline removed (wash No. 1)	0.9% saline solu.	4.8	4.8	4.8	4.8	4.8	4.8
	b. Add 5 ml. saline Mix—centrifuge Saline removed (wash No. 2)	Radioestrone	0.2	0.2	0.2	0.2	0.2	0.2
	c. RBC precipitated							
	Results	Saline wash No. 1	12.6	11.6	9.6	11.2	11.3	10.9
		Saline wash No. 2	9.3	8.2	8.2	8.0	8.0	9.3
		RBC	73.0	75.8	67.5	79.5	63.0	75.4
		Total recovery	94.9	95.6	85.3	98.7	82.3	95.6

twice; it was then centrifuged and the aqueous phase constituted the hemoglobin preparation.

A centrifuge tube was prepared containing 0.1 ml. of radioestrone solution and 5 ml. hemoglobin solution; 5 ml. of washed red cells of Subject D was added and after thorough mixing and centrifugation, the liquid phase was removed. Three more hemoglobin solution washings were carried out. Each of these washings was extracted with chloroform and the red cells were precipitated with ethanol.

The total recovery was 97.4 per cent, the radioactivity left in the red cells was 40.3 per cent and that found in the successive hemoglobin solution washings was 22.2, 15.0, 11.0 and 8.9 per cent. The average distribution coefficient was calculated to be 0.247. It would appear that hemoglobin can remove radioestrone from the red cells faster than 0.9 per cent NaCl solution but not as efficiently as plasma.

Experiment No. 8

To a centrifuge tube containing 0.1 ml. of radioestrone solution and 7.9 ml. of saline solution, 4 ml. of washed red blood cells (Subject E) freed of white cells was added and mixed. The mixture was frozen and thawed twice. It was then centrifuged and the liquid phase was removed and extracted with chloroform. The red cell ghosts, approximately 0.6 ml. in volume, were extracted twice with ethanol.

The liquid phase contained 67.8 per cent of the radioactivity while the solid phase (red cell ghosts) contained 31.5 per cent. It is certain that this latter phase was contaminated with a certain amount of the upper phase. However, if the solid volume had been made entirely of the liquid phase it would have a maximal activity of 3.5 per cent. The red cell ghosts, therefore, had retained a significant amount of radiosteroid. It must also be considered that the liquid phase consisted not only of the red cell fluid but also of 8 ml. of saline solution, representing twice the initial red cell volume. The saline solution of the first washing was found to contain amounts of activity relatively greater than those which could be predicted from the distribution coefficient of the system considered. It follows that hemoglobin was responsible for only a fraction of the 67.8 per cent of the radioactivity detected in the liquid phase.

DISCUSSION

In Experiment No. 1, the calculation of the distribution of radioestrone between plasma and red cells of a given sample is based on the assumption that the value of the microhematocrit gives the real volume of the erythrocytes. When we compared the values of hematocrits obtained by our micro-method and those found with the Wintrobe method (2,080 G for 30 minutes), it was observed that the latter technique gave results 2.4 to 2.9 per cent higher. Since it is generally admitted that the amount of plasma trapped in packed red cells is between 2 and 2.8 per cent of the cell volume obtained by the Wintrobe technique (13, 14), it seems that our assumption was correct. However, theoretically, the percentage of activity calculated to be with the red cells is still probably slightly overestimated.

One could expect that a similar distribution of estrone between plasma and red cells would take place *in vivo;* indeed, we found that following intravenous administration of 16-C^{14}-estrone to three normal adults, the red cells contained 7.9 to 18.7 per cent of the total free activity detected in various blood samples (9). However, since the *in vitro* distribution was immediate and not influenced by temperature in the range of 5° to 37° C. (Experiment Nos. 1 and 2), it is impossible to decide whether this distribution takes place before or after the withdrawal of the blood.

The significance of the *in vivo* studies is limited also by the fact that estrone undergoes extensive metabolism almost immediately after administration and, therefore, the nature of the free steroids of plasma is not necessarily the same as that of the free steroids of red cells.

It is of interest that the red blood cells behaved (Experiment Nos. 3 and 4) as an ordinary solvent for 16-C^{14}-estrone as demonstrated by the existence of a distribution coefficient for the steroid in the system used. It is not surprising that the coefficient would be greater with plasma than saline solution, since it is known that some of the plasma proteins can increase the solubility of steroids (15, 16).

Experiment No. 5 was carried out in order to

study estrone loading of red cells. The results must be considered with some reservation. As pointed out previously, we were not certain that the total amount of nonisotopic estrone was taken up by the red cells. It was found impossible to measure estrone chemically in an extract of red cells because of the presence of interfering chromogens. It was necessary to measure the estrone content of the saline washings and to assume that the amount of steroid which was not in these washings was with the red cells; however, this latter chemical determination was also partly impeded by chromogen interference.

The data presented do not permit us to decide whether estrone was in solution in the intracellular fluid of the red cells or whether it was absorbed on the surface of the cells. The latter possibility seems to be the more probable since an active transfer through a cell membrane is usually influenced by temperature variation; this was not found to be true in Experiment No. 2. Furthermore, estrone had a distribution coefficient in the system saline solution: red cells similar to that found in the system saline solution: stroma (Experiment No. 6) suggesting that the adsorption of radioestrone on stroma could account for the amount of activity associated with the red cells. On the other hand, the addition of hemoglobin to 0.9 per cent NaCl solution increased the distribution coefficient of estrone, showing that hemoglobin itself increases the solubility of the steroid (Experiment No. 7). This might explain why only one-third of the radioestrone was recovered from the red cell ghosts (Experiment No. 8) and, hence, does not necessarily invalidate the theory of surface adsorption of the steroid.

In distribution studies, the compound distributed must not react with the solvents. It has been shown that 4 to 5 per cent of estrone added to whole blood is transformed into estradiol (14). The enzymatic system which produces biological activation requires the presence of glucose, is not destroyed on hemolysis of the red cell and the optimum pH range is 6.6 to 8.2. It is destroyed by heating for one hour at 63° C., by 20 per cent ethanol and by a reaction pH of 4.7 (5). Our experience has been similar (17). However, as pointed out by Bischoff and associates (5), prolongation of the incubation period increases the biological activation and we have observed that the formation of estradiol is insignificant when the period of incubation is 30 minutes or less.

Since 12 to 25 per cent of estrone appears to be associated with the red cells, then the red cells must play a rather important role in the transport of the hormone; the red cells have been found also to produce biological activation of estrone by production of estradiol (5, 14) while plasma alone does not cause such an activation (17). These various findings stress the significance of the study of estrone binding to red blood cells of man. In addition, red cells tagged with radioestrone have been observed to be an interesting tool for the study of the relative binding capacity of various plasma protein fractions (18).

SUMMARY

When 16-C^{14}-estrone was added to blood samples of normal individuals, 75.0 to 88.4 per cent of the radioactivity was recovered in the plasma; the balance of the activity accompanied the red cells. Neither the length of the incubation (0 to 4 hours) nor temperature (5, 22 and 37°C.) appeared to influence this distribution.

The activity accompanying the red cells was slowly removed by successive washings with 0.9 per cent NaCl solution, the average distribution coefficient of 16-C^{14}-estrone for this system being 0.094 to 0.125. The activity was removed much faster by plasma, the average coefficient being 3.09 to 3.29.

It was calculated that the solubility of estrone in red cells was at least 6.08×10^{-5} M per L. and probably greater since overloading was not achieved. The average distribution coefficient of radioestrone between 0.9 per cent NaCl solution and red cell stroma was 0.095, while that between a saline solution of 25 per cent hemoglobin and red cells was 0.247. The suggestion that the phenomena studied were related to adsorption of estrone on the red cell surface was discussed.

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SOME ASPECTS OF THE METABOLISM OF 16-C^{14}-ESTRONE IN NORMAL INDIVIDUALS *

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Until recently, the studies of the physiological disposition of estrogens have been somewhat unsatisfactory because of low recoveries of the administered steroids in urine and feces (1–6). Although recoveries were somewhat increased in investigations in which the steroids were tagged with a radioactive halogen (7, 8) or with deuterium (9), the availability of estrogens tagged with carbon 14 has made possible more precise studies of the physiological disposition of these compounds (10–13).

Beer and Gallagher (10) have reported that the excretion of radioactivity in the urine after injection of estrone-16-C^{14} in two patients was 77 and 58 per cent of the administered dose, while Sandberg and Slaunwhite (12) have reported in nine females an average excretion of 80 per cent of the administered dose, with a range of 41 to 90 per cent. In addition, Sandberg and Slaunwhite, investigating the clearance from plasma of free radioactivity and activity released by β-glucuronidase hydrolysis, noted that a significant amount of activity was associated with the red blood cells (12).

The present paper is concerned with some aspects of the metabolism of 16-C^{14}-estrone when administered to three normal adult subjects, two males and one female. Some of our data are only duplication and confirmation of work already reported in the literature (10–12). However, no study has yet been reported in normal males. Furthermore, in this study an attempt has been made to fractionate and determine the nature of the metabolites of estrone.

METHODS

Experimental subjects. The experimental subjects of this study were two normal males, A. D. and A. N., 32 and 30 years of age, respectively, and a normal 28 year old female (A. K.) who had a regular 29 to 30 day menstrual cycle. This latter subject received the radioactive steroid 11 days after onset of the last menses.

Purity of 16-C^{14}-estrone. The 16-C^{14}-estrone was provided by Charles E. Frosst and Co., Montreal, Canada. Its specific activity was 0.729 mc. per mM. The purity of this material was tested in our laboratory by paper chromatography, using the system of solvents isooctane : toluene : methanol : water (25 : 75 : 80 : 20) of De Courcy (14), and a modified Bush system (15) benzene : skellysolve C® : methanol : water (40 : 60 : 70 : 30). An aliquot of the radioactive steroid was chromatographed and the area of the paper strip corresponding to estrone was eluted; 94 to 98 per cent of the radioactivity of the aliquot was found in the eluate.

Mode of administration of 16-C^{14}-estrone. The steroid was dissolved in redistilled ethanol. An aliquot of this solution was assayed for radioactivity and found to contain 654,000 cpm per ml.

For human administration, a 2 ml. aliquot of the solution was pipetted carefully and diluted with 18 ml. of sterile isotonic saline solution. The methods of intravenous injection and calculation of the dose actually administered were similar to those described for 4-C^{14}-cortisol (16).

The injections were given between 9:30 and 10:30 a.m. over a three minute period.

Mode of collection of samples of blood, urine and feces. Samples of 40 to 50 ml. of blood were collected in heparinized syringes 15, 30, 60, 120, 240 and 360 minutes after the injection of the radioactive material. The blood samples were immediately centrifuged and the plasma separated from the red cells. The red cells were washed twice with twice their volume of isotonic saline solution. It must be noted that the white blood cells were not separated from the red cells.

Urine samples were collected 4, 8, 12, 24 hours following the injection and on the six subsequent days. Feces were collected for 5 to 6 days. All samples were stored in the deep freeze until the time of extraction or counting.

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Method of extraction of the various specimens. Each plasma sample was extracted six times with twice its volume of redistilled chloroform [1] in order to obtain the free steroid fraction. One hundred ml. of absolute ethanol was then added to the plasma residue; after shaking and centrifuging, the liquid phase was decanted and the remainder was washed twice with 50 ml. of ethanol; the pooled extracts were then evaporated to dryness. The ethanol residue was dissolved in 15 ml. of distilled water and incubated with β-glucuronidase [2] (approximately 500 units per ml. of plasma) at pH 4.7 ± 0.1 (acetate buffer), at 37° C. for 48 hours. Extraction was performed with chloroform (30 ml. × 6) to obtain the steroids which had been conjugated with glucuronic acid. The aqueous residue was then subjected to a 48 hour continuous extraction with ether at pH 0.8 to extract the easily hydrolyzable conjugate fraction. Following this procedure, the aqueous residue was boiled for 30 minutes with one-tenth its volume of concentrated HCl and extracted with chloroform to obtain steroids present in plasma as conjugates not hydrolyzed by the preceding procedures.

The red blood cells were placed overnight in the deep-freeze and then extracted with absolute ethanol (100 ml. once and 50 ml. twice). The pooled extracts were then evaporated to dryness. The dry residue was redissolved in 5 to 10 ml. of ethanol and then filtered. This filtrate which represented the total red cell extract was evaporated to dryness. The residue, dissolved in 10 ml. of distilled water, was extracted with chloroform (20 ml. × 6) to obtain the free fraction of the red cell extract. The aqueous residue was then incubated with β-glucuronidase and extracted with chloroform (30 ml. × 6); this constituted the glucuronoside fraction [3] of the red cell extract.

A 25 ml. aliquot of each urine sample was extracted six times with twice its volume of chloroform to obtain the free steroids. The residue was subjected to β-glucuronidase hydrolysis, followed by a 48 hour continuous extraction with ether at pH 0.8, and subsequent hydrolysis with HCl.

Each feces collection was extracted five times with 95 per cent ethanol (3,000 ml.) using a Waring blendor for mixing. The ethanol extracts were separated from feces by centrifugation and filtration. The ethanol was evaporated to dryness and the residue was dissolved in 100 ml. water. This aqueous fraction was extracted five times with twice its volume of chloroform to obtain the free steroids and then β-glucuronidase hydrolysis was carried out.

Radioactivity determinations. All extracts of plasma, red blood cells, urine and feces, as well as whole urine, were assayed for radioactivity in the manner previously described (16).

Benzene-water fractionations. Some of the plasma and urine extracts were evaporated to dryness. The respective residues were dissolved in 15 ml. of redistilled benzene and the benzene was extracted three times with its volume of distilled water.[4]

Paper chromatography. Some of the residues resulting from a benzene : water fractionation were chromatographed on paper using the systems of solvents isooctane : toluene : methanol : water (25 : 75 : 80 : 20) for the benzene fraction, and benzene : methanol : water (100 : 55 : 45) for the water fraction. The R_f values of various estrogen standards [5] when using the two systems described above and a third system of solvents, benzene : skellysolve C® : methanol : water (40 : 60 : 70 : 30), are shown in Table I.

The estrogens on paper chromatograms were visualized either by coupling with diazotized sulfanilic acid as described by Berry, Sutton, Cain and Berry (17) or by the method of Mitchell (18).

Radioautography. Some of the paper chromatograms were radioautographed using Kodak Medical X-Ray films (tinted, duplitized, no screen). On most occasions the time of exposure was three weeks. Following this procedure the areas of the papergram corresponding to the darkened zones of the radioautogram were excised and the pieces obtained were placed in an Erlenmeyer flask. After the addition of absolute ethanol (10 to 15 ml.) the flask was agitated by means of an automatic shaker for 30 minutes. The procedure was repeated twice more using ethanol and finally once using chloroform. The pooled eluates were filtered, evaporated to dryness and the radioactivity of the residue was determined.

RESULTS

1. *Plasma studies*

The amount of radioactivity readily extractable from plasma by chloroform and that obtained fol-

[1] When using this method of extraction, pure estrone and estradiol-17β added to water were quantitatively recovered while 90 to 94 per cent of the added estriol was recovered.

[2] Ketodase, a beef liver preparation, from Warner-Chilcott Laboratories was used in the present study.

[3] The correct term to designate this type of steroid conjugate is "steroid glucusiduronic acid." However, the colloquial terms "glucuronide" and "glucuronoside" are widely used. In the present paper, "glucuronoside fraction" is used to designate the group of steroids which are liberated by β-glucuronidase hydrolysis.

[4] When pure estrone, estradiol-17β, 16-epiestriol and estriol were submitted to such a fractionation, it was found that an average of 98.5 per cent of the estrone, 95 per cent of the estradiol-17β and 90.4 per cent of the 16-epiestriol could be recovered in the benzene fraction, while 88.5 per cent of the estriol was in the water fraction.

[5] The authors are very grateful to Dr. Max N. Huffman, Professor of Research Biochemistry, Oklahoma Medical Research Institute, Oklahoma City, who kindly provided the following steroid standards: 1,3,5(10),16-estratetraen-3-ol, estrone-16, estradiol-16β, estradiol-16, 16-ketoestrone, 16-ketoestradiol-17β and 16α-epiestriol. Thanks are also due to the Parke, Davis and Company who provided us with estrone and estradiol-17β standards.

TABLE I

R_f values of several estrogens for three different systems of solvents

Estrogens	Systems of solvents		
	Isooctane:toluene: methanol:water (25:75:80:20)	Benzene:Skelly C®: methanol:water (40:60:70:30)	Benzene: methanol:water (100:55:45)
Estriol	0.025	0	0.090
16-Epiestriol	0.156	0.018	0.438
16-Ketoestradiol	0.311	0.084	0.640
16-Ketoestrone	0.488	0.167	0.752
Estradiol-16α	0.494	0.215	0.786
Estradiol-17β	0.536	0.279	0.786
Estradiol-16β	0.567	0.265	0.808
Estrone	0.780	0.648	0.878
Estrone-16	0.802	0.660	0.902
1,3,5(10),16-estratetraene-3-ol	0.890	0.920	0.922

lowing the various hydrolysis procedures are shown in Table II. The results are expressed as percentage of the administered dose per 1,000 ml. of plasma.

Fifteen minutes after the injection of 16-C^{14}-estrone, only 2.76 to 3.79 per cent of the dose was present as free steroid in the total plasma volume (estimated as 5 per cent of body weight). The amount of radioactivity in the glucuronoside fraction from plasma was maximal 60 to 120 minutes after the injection and did not significantly change during the next few hours. The fractions obtained from continuous ether extraction at pH 0.8 and strong acid hydrolysis contained a small, but statistically significant, amount of radioactivity. The trend of these values was somewhat similar to that observed for the values of the glucuronoside fraction (Table II). These data differ from those reported by Sandberg and Slaunwhite (12) only in respect to the elevation in the glucuronoside fraction which in the present study appeared more sustained.

The activity in the water extract following benzene : water partition of the free and glucuronoside fractions from plasma of Subjects A. D. and A. K. was found to represent only a small percentage of the total activity (free fraction: 3.1 to 4.2 per cent for Subject A. D., 6.2 to 7.3 per cent for Subject A. K.; glucuronoside fractions: 1.4 to 7 per cent for A. D., 4.8 to 9.3 per cent for A. K.).

TABLE II

Radioactivity in the plasma and washed red blood cells of three normal subjects following 16-C^{14}-estrone administration (expressed as percentage of the administered dose per liter of plasma)

Subjects	Min. after inject.	Per cent dose/1,000 ml. Plasma				Red cells		
		Free	Gluc.	Continuous ether ext. pH 0.8	Strong acid hydro.	Total	Free	Gluc.
A. D.	15	0.79	0.28	0.02	0.00	0.50	0.12	0.03
	30	0.51	0.71	0.04	0.01			
32 yrs.	60	0.40	0.78	0.03	0.02			
	120	0.19	0.90	0.06	0.12	0.28	0.06	0.00
70 Kg. ♂	240	0.15	0.86	0.06	0.09			
A. K.	15	1.35	0.30	0.07	0.05			
	30	0.81	0.49	0.04	0.04	0.67	0.12	0.08
28 yrs.	60	0.53	0.80	0.06				
	120	0.14	1.25	0.10	0.08			
56 Kg. ♀	240	0.08	0.86	0.11	0.13	0.31	0.03	0.02
	360	0.04	0.77	0.05	0.16			
A. N.	15	0.72	0.50	0.08	0.03	0.75	0.17	0.05
	30	0.52	0.66	0.07	0.04			
30 yrs.	60	0.30	0.72	0.07	0.04			
	120	0.12		0.14	0.08	0.46	0.05	0.01
78 Kg. ♂	240	0.10	0.60	0.24	0.12			
	360	0.06	0.92	0.08	0.09			

TABLE III

Distribution between plasma and red cells of the radioactivity (total, free, glucuronoside) of 1,000 ml. of whole blood

Subject	Min. after injection	Hematocrit (av.)	% Dose in plasma 1,000 ml. blood			% Dose in RBC 1,000 ml. blood		
			Total*	Free	Gluc.	Total	Free	Gluc.
A. D.	15–30	43	0.672	0.370	0.282	0.217 (24.4%)†	0.052 (12.3%)	0.011
	60–120–240	42	0.705	0.143	0.487	0.115 (14.0%)	0.023 (13.9%)	0.000
A. K.	15–30–60	40	0.835	0.537	0.238	0.268 (24.3%)	0.046 (7.9%)	0.030
	120–240–360	39	0.762	0.052	0.586	0.119 (13.5%)	0.012 (18.7%)	0.008
A. N.	15–30	45	0.710	0.342	0.307	0.337 (32.2%)	0.076 (18.2%)	0.024
	60–120	43	0.703	0.121	0.490	0.198 (22.0%)	0.023 (16.0%)	0.006

* Total in plasma is average of sum of various plasma fractions of Table II.
† (RBC activity/plasma activity + RBC activity) × 100.

When the benzene extract of the free fraction was chromatographed on paper, the radioactivity present in the area of the papergram corresponding to estrone constituted a greater percentage of the total activity in the first plasma samples than it did in subsequent ones (85 and 87 per cent of the benzene fraction in the 15 minute samples of Subjects A. D. and A. K., decreasing progressively to 45 and 42 per cent in the 240 minute samples).

2. Red blood cell studies

Substantial amounts of activity were associated with the red cells (Table II), confirming the finding of Sandberg and Slaunwhite (12).

The total activity in 1,000 ml. of whole blood was calculated on the basis of the hematocrit and of the sum of the activity in the various plasma fractions and red cell extracts. From this, it was determined that 13.5 to 32.2 per cent of the total activity in a liter of blood was associated with the red cells (Table III).

A similar calculation was made for the radioactivity readily extractable by chloroform. As seen in Table III, 7.9 to 18.7 per cent of the free steroids in a liter of blood was associated with the red cells.

The glucuronoside fraction from red cells was very small so that a large amount of the total radioactivity in red cells was not accounted for. In Subject A. D., the residues obtained after removal of the free and glucuronoside fractions from the total red cell extracts contained 0.341 per cent of the dose per 1,000 ml. of red cells for the 15 to 30 minute samples and 0.200 per cent for the 60 to 240 minute samples. These data would tend to show that an important proportion of the compounds found in red cell extracts is in a state other than as free steroids or their glucuronosides.

3. Urine studies

The data on the urinary excretion of radioactivity are shown in Table IV. From 79.9 per cent (Subject A. D.) to 89.5 per cent (Subject A. K.) of the administered dose was recovered during the first six days following the injection of 16-C^{14}-estrone. No significant amount of radioactivity could be detected after this time. These data are in accordance with the results published by other investigators (10, 12).

The unaccounted-for fraction of urinary radioactivity. A rather large amount of urinary radioactivity was unaccounted for in all cases, and some attempts were made to determine whether this was due to technical error. The following experiment was carried out:

At the end of the first 24 hours, 51.10 per cent of the administered dose was excreted in the urine of Subject A. K.; of this, 40.82 per cent was recovered in the free fraction plus the fractions obtained after β-glucuronidase hydrolysis and con-

tinuous extraction at pH 0.8 (see Table IV). Following these procedures the radioactivity in the urine residue was 6.50 per cent of the administered dose, and therefore there was a net loss of 3.78 per cent, representing the sum of the experimental errors (handling and counting of each individual sample) to that point.

Benzene:water partition of the various urinary fractions. The benzene:water partition of the various urinary fractions is shown in Figures 1 and 2. The percentage of activity found in the benzene phase was relatively constant for the free fraction of the various urine collections and represented 40 to 60 per cent of the total activity (Figure 1). In the glucuronoside fraction, the percentage of activity in the benzene phase was 75 to 90 per cent of the total in the 0 to 4 hour urine specimens, but this percentage had a definite tendency to decrease with time and was only 42 to 60 per cent of the total in the 48 to 72 hour urine collection. A similar but less marked variation was observed for the pH 0.8 hydrolysis fraction as well as the strong acid hydrolysis fraction (Figure 2).

Paper chromatography of the urinary glucuronoside fractions. The urinary metabolites freed by β-glucuronidase hydrolysis were partitioned between benzene and water. Each fraction was chromatographed on paper and a radioautogram of the papergram was prepared.

The results obtained with the water extract of the various samples was difficult to interpret because of unsatisfactory separation of the compounds present in this fraction. However, it was observed that: *1*) the radioautograms showed a very faint spot corresponding to 16-epiestriol but there were no spots corresponding to compounds less polar than 16-epiestriol; *2*) a spot corresponding to estriol was detected in every instance; but, *3*) there was also a large amount of material which had not moved from the origin, and therefore was more polar than estriol.

The radioautograms of the benzene extracts showed a series of spots: *1*) a spot representing

TABLE IV

Cumulative urinary excretion of radioactivity following injection of 16-C^{14}-estrone (expressed as percentage of the administered dose)

Subject	Time after inject.	Per cent dose excreted in urine					
		Total	Free	β-Gluc.	Cont. extract.	Strong acid	Unaccounted
A. D.	4 hrs.	5.56	0.10	4.25	0.58	0.28	0.35
	8 hrs.	11.55	0.15	7.51	1.36	0.59	1.94
	12 hrs.	17.07	0.20	11.39	1.84	0.84	2.80
	1 day	37.81	0.45	22.52	3.57	1.40	9.87
	2 days	63.30	0.83	38.57	6.02	2.18	15.70
	3 days	71.10	1.00	43.50	6.81	2.53	17.26
	4 days	74.40					
	5 days	78.30					
	6 days	79.90					
	7 days	79.90					
A. K.	4 hrs.	8.28	0.19	5.88	0.82	0.44	0.95
	8 hrs.	17.41	0.35	11.92	1.49	0.90	2.75
	12 hrs.	26.03	0.49	18.50	2.02	1.40	3.62
	1 day	51.10	0.74	36.35	3.73	2.63	7.65
	2 days	71.95	0.93	52.60	5.30	3.75	9.37
	3 days	80.70	0.99	57.00	5.59	4.09	13.03
	4 days	86.20					
	5 days	88.90					
	6 days	89.50					
	7 days	89.50					
A. N.	4 hrs.	6.58	0.17	3.25	0.94	0.56	1.66
	8 hrs.	15.04	0.34	7.66	1.92	1.45	3.67
	12 hrs.	22.22	0.49	11.18	2.61	1.98	5.96
	1 day	37.40	0.72	20.30	3.48	2.75	10.15
	2 days	59.40	1.06	33.35	4.87	3.51	16.61
	3 days	71.00	1.28	40.30	5.59	3.89	19.94
	4 days	78.40					
	5 days	79.80					
	6 days	80.60					
	7 days	80.60					

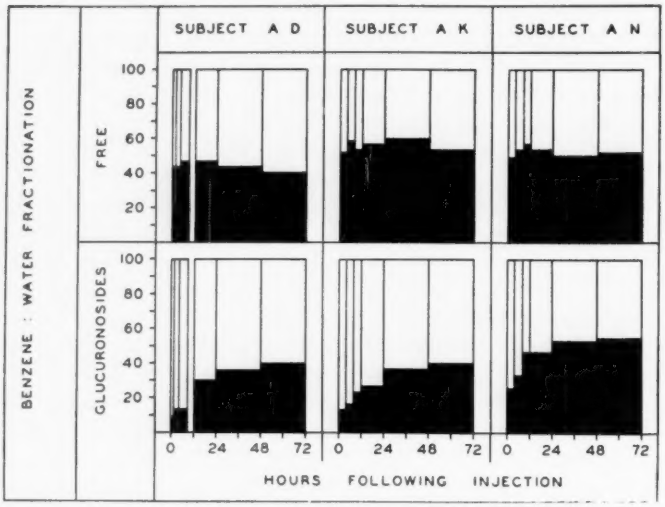

FIG. 1. PARTITION BETWEEN BENZENE AND WATER OF THE FREE AND GLUCURONOSIDE FRACTIONS OF THE VARIOUS URINE COLLECTIONS

The activity in benzene and in water are expressed as per cent of the sum of the activity of both phases. The black columns represent the water fraction.

the least polar compound with an R_f value of approximately 0.850, *2*) a large spot of R_f value 0.775 to 0.790, corresponding to estrone, *3*) a spot of R_f value 0.545 to 0.530, corresponding to estradiol-17β, *4*) a spot of R_f value 0.495 to 0.480 corresponding to either estradiol-16α or more probably 16-ketoestrone, *5*) a spot of R_f value 0.305 to 0.315, corresponding to 16-ketoestradiol or pos-

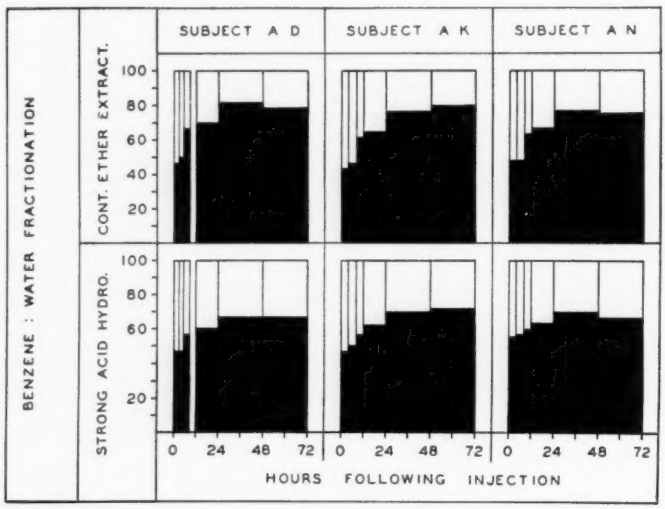

FIG. 2. PARTITION BETWEEN BENZENE AND WATER OF THE PH 0.8 HYDROLYSIS FRACTION AND OF THE STRONG ACID HYDROLYSIS FRACTION OF THE VARIOUS URINE COLLECTIONS

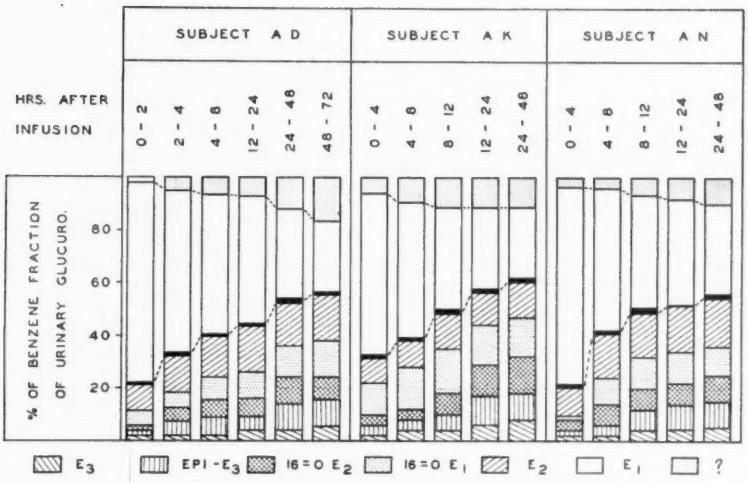

FIG. 3. BENZENE FRACTION OF A BENZENE: WATER PARTITION OF THE URINARY GLUCURONOSIDES

This fraction was chromatographed on paper and a radioautogram was prepared. The activity of each spot on the papergram is expressed as per cent of the sum of the activity obtained for the various spots. Each column represents the results obtained for the various urine samples collected from the experimental subjects. The black section of the columns represent the percentage of activity found in the area of the papergrams situated between the spots of estrone and estradiol-17β. The closely stippled section at the top of each bar represents the material with an R_f value of 0.850.

sibly 16-hydroxyestrone, *6*) a spot of R_f value 0.150 to 0.161 corresponding to 16-epiestriol, *7*) and finally, a small amount of material very close to the origin corresponding to estriol.

A radioautogram of the benzene extract of each of the urine collections was prepared. The corresponding spots of the chromatograms were excised carefully and the material present on each of these pieces of paper was eluted using 100 per cent ethanol as eluant. The radioactivity of each eluate was determined. Figure 3 presents the values obtained for each eluate expressed as percentage of the sum of the values obtained for the various spots. The recoveries were 91 to 106 per cent of the material applied on paper for chromatography.

Partial identification of some of the spots observed on the radioautograms of the benzene extract of the urinary glucuronoside fractions. The compound of R_f 0.850 and that corresponding to estriol were not studied. A known quantity of the urine collected between four and 48 hours after the administration of 16-C^{14}-estrone to Subject A. K. was extracted with $CHCl_3$ to remove the free steroids. The urine residue was treated with β-glucuronidase and the steroids liberated by this procedure were extracted with $CHCl_3$. This extract was partitioned between benzene and water and the benzene extract was chromatographed on several paper strips. Radioautograms of these papergrams were prepared, and the areas of the papergrams corresponding to estrone, estradiol-17β, 16-ketoestrone, 16-ketoestradiol-17β and 16-epiestriol were extracted with ethanol. Paper chromatography of the pooled extracts of each individual compound was repeated until the respective radioautograms showed only one well defined spot suggesting the presence of a single compound in each case.

The eluates of each spot were then chromatographed individually on an 11 × 70 mm. column of florisil. The florisil was activated as described for the determination of plasma 17-hydroxycorticosteroids (19). The dry residues from the eluates of estrone and estradiol-17β were transferred on the column with 20 ml. of chloroform, and the steroids were eluted with 60 ml. of 0.4 per cent methanol in chloroform. 16-Ketoestrone,

TABLE V

*Specific activity following recrystallization of some of the urinary metabolites of 16-C^{14}-estrone**

Compounds	Initial	Recrystallization		
		First	Second	Third
Estrone	5,070	4,850	4,960	4,740
Estradiol-17β	3,120	3,070	2,890	3,090
16-Keto-estrone	3,010	2,900	3,110	2,820
16-Keto-estradiol	1,945	1,630	1,290	1,070
16-Epiestriol	1,540	1,490	1,505	1,385

* The compounds studied were those freed by β-glucuronidase hydrolysis present in the benzene fraction of a benzene : water fractionation.

(Fractionation. All values expressed as cpm per mg.)

16-ketoestradiol-17β and 16-epiestriol were transferred on columns with 70 ml. of chloroform; then, 20 ml. fractions of 0.1 per cent, 0.2 per cent and 0.5 per cent methanol in chloroform were successively added to the column and discarded, the steroids being eluted with 50 ml. of 2 per cent methanol in chloroform.

The radioactivity present in the final residues was determined. A carefully weighed amount of pure crystalline steroid (19.5 mg.) was added to the presumably corresponding compound. Repeated recrystallization from aqueous methanol showed the specific activity to be constant for estrone, estradiol-17β, 16-ketoestrone and 16-epiestriol (Table V). However, the specific activity for the 16-ketoestradiol-17β fraction had a tendency to decrease, indicating that we were not dealing with 16-ketoestradiol-17β or, at least, not in pure form.

In addition, a 25 transfer countercurrent distribution with the system of solvents methanol: water : carbon tetrachloride (50 : 50 : 100) for estrone and estradiol and the system methanol : water : carbon tetrachloride (20 : 80 : 100) for 16-ketoestrone and 16-epiestriol was carried out on the various samples; the radioactivity was found to distribute as the corresponding standard steroids.

An identical study was carried out on the urine of Subject A. N. with similar results.

4. Feces studies

The three experimental subjects excreted 8 to 14 per cent of the injected dose within five days following the injection of 16-C^{14}-estrone. One-half or more of this activity was directly extractable by chloroform, but only a small fraction of the fecal radioactivity (0.21 to 0.58 per cent of the administered dose) was liberated following β-glucuronidase. These results are in good agreement with those published by others (12).

DISCUSSION

The results of the present study of the physiological disposition of 16-C^{14}-estrone in two normal males and one female are similar in most respects to those reported by other workers (10, 12) who have confined their investigation to female subjects. Estrone was found to disappear very rapidly from the blood stream, since during the first 15 minutes following the injection of the radioactive steroid only approximately 5 per cent of the administered dose remained in the circulation. After this time, however, the free activity disappeared from plasma at a progressively slower rate while the high levels of the glucuronoside fraction were sustained during the entire period.

As previously noted by Sandberg and Slaunwhite (12), significant amounts of radioactivity appeared to be associated with the red blood cells. *In vitro* studies of the distribution between plasma and red cells of 16-C^{14}-estrone have shown that 11.6 to 25 per cent of the total blood activity was recovered in the red cell extracts (20). Similar results have been reported by other investigators (21, 22). These values are similar to those calculated for the free red cell activity in this work. Since the distribution *in vitro* was found to be very rapid and not influenced by temperature (20) it is difficult to decide whether the distribution takes place before or after the withdrawal of the blood.

Only 10 to 20 per cent of the total activity detected in red cells was readily extractable by chloroform, suggesting that a large fraction of this total activity was in a conjugated form. This finding is in contrast with *in vivo* studies carried out with cortisol where it was observed that none of the conjugated metabolites of cortisol were associated with the red cells (23). Only very small amounts of activity were liberated by β-glucuronidase hydrolysis and since no attempt was made to further hydrolyze the conjugated steroids associated with the red cells, it is impossible to determine in what form the remaining radioactivity occurred.

Several metabolites of estrone were detected in the benzene extract of the urinary glucuronoside fractions. Our identification of 16-ketoestrone confirms the results of Slaunwhite and Sandberg (24). Levitz, Spitzer and Twombly (13) have demonstrated the conversion of estradiol-17β-16-C^{14} into 16-ketoestradiol-17β in man. Marrian, Loke, Watson and Panattoni (25), who have isolated 16α-hydroxyestrone from pregnant women's urine, pointed out that this latter compound readily undergoes rearrangement in alkaline solution to 16-keto-estradiol-17β. One of the compounds detected in the present work appeared to be 16-keto-estradiol-17β; however, it did not have a constant specific activity and possibly represented a mixture of the isomers of 16α-hydroxyestrone and 16-ketoestradiol-17β. It must be noted that our glucuronoside fraction was studied directly without phenolic fractionation. The 16-epi-isomer of estriol has been isolated from the urine of pregnant women (26) and has been found also in the urine of the three subjects of the present study as one of the metabolites of estrone. It is of interest that these various metabolites of estrone had previously been detected on the paper chromatograms of a nonhomogenous "fraction E_4" prepared from urinary extracts of pregnant women (27). Finally, the spot of R_f value 0.850 which appeared on our radioautograms could possibly be 2-methoxyestrone recently isolated in man as a new metabolite of estradiol-17β by Kraychy and Gallagher (28). A spot of R_f value slightly greater than that of estrone had previously been detected in the urine extracts of pregnant women (27) which possibly could be the same compound, 2-methoxyestrone.

Beer and Gallagher (11) have noted that following administration of estradiol-17β the amount of estriol recovered from urine increased with time. Comparable results were obtained in the present study; furthermore, it was also true for the other metabolites of estrone. It is possible that this dynamic change in the relative proportion of the various metabolites is due to the fact that estrone undergoes extensive entero-hepatic circulation (12) and, therefore, has a better chance to be metabolized by either repeated perfusions through the liver or in the gut.

In recent years, chemical assay methods for estrogens naturally occurring in human urine have been developed (29, 30). These methods were demonstrated to be quite sensitive and specific. Using such techniques, it was observed that one could recover in the urine of the experimental subjects 22 per cent of the injected estrone, 9.5 per cent of the dose being identified as estrone, 2.4 per cent as estradiol-17β and 10.1 per cent estriol (31). Our work and other isotopic studies (10, 12) show that two-thirds to three-fourths of the administered dose of estrone can be recovered in the urine. Nevertheless, as can be seen in Table VI, the percentages of the dose recovered in the urinary glucuronoside fraction as estrone (8.36 to 14.75 per cent) and estradiol-17β (2.95 to 4.94 per cent) were not very different from those reported by Brown (31). It must be noted that our figures do not include the amounts of estrone and estradiol freed by continuous ether extraction at pH 1.0 and by strong acid hydrolysis. On the other hand, the new chemical methods of estrogen determination do not measure certain compounds present in the urine in significant amounts (Table VI) since some of these compounds such as 16-ketoestrone do not give any color with the Kober reagents. Furthermore, the percentage of estriol in Brown's work is only one-half of the sum of our water fractions, but as mentioned in the Results section the water fraction is not homogenous and probably contains, in addition to estriol, the various estriol isomers and perhaps compounds more oxygenated than estriol.

We believe that isotopic estrogen studies of the type presented in this paper will help in improving present chemical methods of estrogen determination by indicating the ideal recoveries that might be expected and the nature of the metabolites to be measured.

SUMMARY

The physiological disposition of 16-C^{14}-estrone administered intravenously has been studied in three normal subjects (two males and one female). The results of blood, urine and feces studied were in accordance with those reported by Beer and Gallagher (10) and Sandberg and Slaunwhite (12).

Investigation on the nature of the urinary metabolites of radioestrone were carried out: 59.40 to 71.95 per cent of the administered dose was excreted in 48 hours with only 42.79 to 62.58 per cent of the administered dose accounted for after the various techniques of hydrolysis. Ap-

TABLE VI

Calculated amounts of some of the urinary metabolites of 16-C^{14}-estrone

Urinary activity excreted during the first two days following 16-C^{14}-estrone injection			Experimental subjects		
			A. D.*	A. K.	A. N.
Total activity			71.10	71.95	59.40
Activity accounted for†			53.84	62.58	42.79
Sum of water fractions‡			37.51	19.31	20.22
	Total		43.50	52.60	37.35
	Water fraction		12.91	13.61	14.73
Gluc. fraction	Benzene fraction	E_3§	1.15	2.11	0.75
		16-Epi-E_3	2.18	3.19	1.40
		16-Keto-E_2 (?)	2.18	3.94	1.53
		16-Keto-E_1	3.17	5.85	1.82
		E_2	4.94	4.86	2.95
		E_1	13.67	14.75	8.36
		2-Methoxy-E_1 (?)	2.78	3.97	1.37

* Urinary activity excreted during the first three days following 16-C^{14}-estrone injection.
† Sum of radioactivity in free, glucuronoside, pH 1.0 hydrolysis and strong acid fraction.
‡ Sum of the water fractions of the free, glucuronoside, pH 1.0 hydrolysis and strong acid fractions.
§ E_3, estriol and compounds more polar than estriol; 16-epi-E_3, 16-epiestriol; 16-keto-E_2, compounds which had R_f value similar to that of 16-ketoestradiol; 16-keto-E_1, 16-ketoestrone; E_2, estradiol-17β; E_1, estrone; 2-methoxy-E_1, compound which is possibly 2-methoxyestrone.

proximately one-half of the latter values represented metabolites as polar as, or more polar than, estriol. The urinary glucuronoside fraction consisted of: estrone (8.36 to 14.75 per cent of the administered dose), estradiol (2.95 to 4.94 per cent), estriol and more polar compounds (14.06 to 15.72 per cent), 16-ketoestrone (1.82 to 5.85 per cent), 16-epiestriol (1.40 to 3.19 per cent), a mixture of the isomers of 16-ketoestradiol and 16α-hydroxyestrone (1.53 to 3.94 per cent) and a compound which could be 2-methoxyestrone representing 1.37 to 3.97 per cent of the administered dose.

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ZONE ELECTROPHORETIC STUDIES OF SERUM ALKALINE PHOSPHATASE * †

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The serum alkaline phosphatase activity is increased in bone diseases associated with increased osteoblastic activity and in certain disorders of the liver and biliary tract, particularly those characterized by biliary tract obstruction or by hepatic infiltration with neoplastic or granulomatous processes (1, 2). Although there is much experimental evidence in support of the view that bone is the source of the serum alkaline phosphatase and that the bile serves as an important channel for its excretion (3, 4), the hepatic retention theory fails to provide a completely satisfactory explanation for the occurrence, in certain hepatobiliary disorders, of increased serum phosphatase activity without hyperbilirubinemia, nor does it account for the normal serum phosphatase values found in many cases of severe parenchymatous liver disease. It has been suggested on the basis of experimental (5) and clinical (6) observations that under certain circumstances the liver may, by its secretory activity, actively contribute to the increase in serum alkaline phosphatase. Although studies of the kinetics of the serum phosphatase activity (7) and of the effects of enzyme inhibitors (8) have failed to reveal differences between the serum phosphatase activity in cases of bone and biliary disease, it seemed of interest to explore further the possible occurrence of qualitatively distinct serum alkaline phosphatases. Since zone electrophoretic methods have proven useful in a variety of enzyme separations, a study was made by this technique of the alkaline phosphatase patterns of sera obtained from patients suffering from disorders associated with increased serum phosphatase activity. The results suggest the occurrence in some cases of more than one serum alkaline phosphatase.

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† An abstract describing a portion of this study has been published (J. clin. Invest. 1957, **36**, 924).

MATERIALS AND METHODS

Most of the cases studied were observed in the wards of the Boston City Hospital. The clinical diagnoses were supported in many instances by anatomic study of surgical, biopsy or necropsy material.

Venous blood samples from the patients were obtained without regard to the time of day or recent ingestion of meals. After the blood had clotted, the separated serum was stored at 4° C. and generally used for electrophoretic study within a few days; in many cases, serum samples were stored frozen and used weeks or months later. Electrophoretic patterns of protein and of enzymatic activity were little affected by such storage, as compared with fresh, unfrozen serum. Zone electrophoresis of serum was performed with starch blocks as the supporting medium, the general technique described by Kunkel (9) being used. The starch blocks were prepared from slurries of potato starch which prior to use had been repeatedly washed by decantation in pH 8.6 barbital buffer. Matheson, Coleman and Bell and Merck brands of starch were satisfactory. The blocks were prepared in a rectangular lucite trough ($40 \times 10 \times 2$ cm.) lined with wax paper; the trough also supported the blocks during electrophoresis. Usually three parallel blocks, each containing 70 Gm. of starch and each 35 cm. long, 3.3 cm. wide, and 1 cm. thick, were prepared in the same trough, the sides of their wax paper envelopes separating one from the other, and electrophoresis of three serum samples was performed simultaneously. For preparative work a single large block (210 Gm. starch and 10 cm. wide) was used. Buffer-saturated plastic sponges and filter paper strips provided electrical continuity between the blocks and the buffer vessels, and the latter were connected by buffer-filled inverted U-tubes to the vessels containing platinum electrodes. The buffer was barbital-sodium barbital, pH 8.6, ionic strength 0.1. Test samples, diluted with an equal volume of buffer, were delivered from the needle of a syringe to a transverse trough (approximately one mm. wide) cut in the starch block midway between the center and cathode end. The volume of material applied was 0.7 ml. in the case of the small blocks, and 3.5 to 5 ml. for the larger blocks. The solution in the buffer vessels was used repeatedly for a number of experiments before being discarded, the polarity of the electrodes being reversed after each run. The starch block and associated vessels were kept in a refrigerator at 4° C. throughout the period of electrophoresis. A potential difference of 2.5 to 3 volts per cm. was maintained between

the ends of the block and the current was 1.4 mA. per cm. width.

At the end of the electrophoretic period of 36 to 48 hours, which under these conditions permitted albumin migration of 15 to 20 cm., the block was sectioned transversely into serial segments 5 mm. wide from a point 1 or 2 cm. on the cathode side of the origin to a point just beyond the albumin zone, which was generally visible because of its yellowish color (9). A section to be used as a blank was taken from a region close to the cathode end of the block well outside the range of protein migration. Each starch segment was suspended in 5.0 ml. water, the mixture was shaken gently and then filtered through Munktell 00 paper. Aliquots of the filtrates were analyzed for proteins (10) and for alkaline phosphatase by the nitrophenylphosphate method of Bessey, Lowry and Brock (11). For phosphatase determination 0.5 to 2.0 ml. of filtrate was added to an equal volume of buffered substrate solution and incubated at 38° C. for 30 to 120 minutes, depending upon the activity of the samples. Aliquot volumes and incubation times were the same for all sections of a particular block. At the end of the incubation period, NaOH solution (0.05 to 0.10 M) was added to each tube to make the total volume 7 ml. and the optical density measured at 410 mμ (Coleman Junior spectrophotometer) before and after acidification with two or three drops of concentrated HCl. From the calibration curve relating *p*-nitrophenol concentration to optical density, phosphatase activity was expressed as μM nitrophenol liberated per hour per ml. filtrate. The buffer-substrate solution was a mixture of equal volumes of 0.3 per cent aqueous solution of *p*-nitrophenylphosphate disodium (Aldrich Chemical Co.) and of pH 10.3 buffer and contained magnesium chloride at a concentration of 5×10^{-4} M. In the earlier experiments 0.1 M glycine-NaOH was used as the buffer (11), while in the later work the more satisfactory 2-amino 2-methyl-1-propanol-HCl (0.5 M) was used (12). Comparison of phosphatase and protein zones was made by plotting the phosphatase and protein concentration of each filtrate as ordinates and the migration distances as abscissa. The completeness of recovery of phosphatase activity from the block after serum electrophoresis is discussed under Results.

Acid phosphatase activity in the eluates of several starch blocks was determined by incubating aliquots with equal volumes of a mixture of *p*-nitrophenylphosphate solution and acetate buffer, pH 5.4 (13). In three experiments I^{131}-labeled thyroxine (Abbott) was added to serum samples prior to electrophoresis and the distribution of radioactivity in the block subsequently determined by measuring aliquots of the filtrate in a well-type scintillation counter.

Serum alkaline phosphatase activity was determined by the King-Armstrong method (14). Serum protein-bound hexose was determined by the orcinol method described by Winzler (15).

RESULTS

The electrophoretic patterns showed that in every case the major zone of alkaline phosphatase activity was in the region of the alpha-2 globulins; the peak of activity sometimes coincided with this protein peak and sometimes the mobility of the active material was slightly less than that of the alpha-2 protein peak. In some cases, particularly those with hepatobiliary disease, a second zone of phosphatase activity was present in the region of the alpha-1 globulins. In general no phosphatase was detected in the gamma globulin or albumin zones. Phosphatase activity was usually present in the beta-globulin zone of the electrophoretogram whence the activity increased in the anodeward direction to form the ascending limb of the alpha-2 activity peak. Occasionally a broad plateau of appreciable activity was seen in the beta globulins and in several instances the occurrence of a zone of slightly decreased activity between this region and the alpha-2 activity suggested the presence of a beta phosphatase zone with a peak.

Since the alpha-2 and alpha-1 components of phosphatase activity appeared to be the most prominent, quantitative analysis of the electrophoretic phosphatase patterns was made on the basis of the relative proportions of these components. This was done by dividing the total phosphatase activity in the graphical representation of the electrophoretic patterns into what will be called "alpha-2" and "alpha-1" fractions. In those patterns in which two distinct phosphatase peaks were present, a perpendicular was drawn from the lowest point of the trough between these zones, and the phosphatase activity on the anode side of the line was considered to be alpha-1 activity, while that on the cathode side (which comprised the remainder of the total activity) was called the "alpha-2" fraction. In those cases where no distinct alpha-1 activity peak was apparent, but where the descending anodeward limb of the alpha-2 zone encroached upon the alpha-1 globulin region, a perpendicular was dropped from the nadir of the trough between alpha-2 and alpha-1 globulins, and division into alpha-2 and alpha-1 activities was made by considering activity on the anode side of the line to be "alpha-1." In effect, all the phosphatase activity of mobility less than that of the alpha-1 globulins was called the "alpha-2" fraction.

Table I summarizes the results in 63 cases studied by starch block electrophoresis; the di-

TABLE I

Clinical data and electrophoretic phosphatase patterns of cases studied

Case no.	Age	Sex	Diagnosis	Serum alkaline phosphatase (King-Armstrong)	Number of phosphatase peaks	Per cent of activity in	
						alpha-2	alpha-1
1	42	F	Ovarian carcinoma, hepatic metastases	37	2	85	15
2*	82	M	Gastric carcinoma, hepatic metastases	107	2	89	11
3*	75	F	Gallbladder carcinoma, hepatic metastases; cholangitis	30	2	87	13
4*	55	M	Hepatic duct carcinoma, hepatic metastases, biliary cirrhosis	373	2	68	32
5*	79	F	Carcinoma of colon, hepatic metastases	150	2	56	44
6*	44	F	Carcinoma of breast, hepatic metastases	123	2	81	19
7*	63	M	Hepatic carcinoma, primary site undetermined, pigment cirrhosis	51	2	88	12
8*	73	M	Sigmoid carcinoma, hepatic metastases	78	2	89	11
9*	75	F	Gall bladder carcinoma, hepatic metastases	242	2	80	20
10*	81	M	Hepatic duct carcinoma, biliary cirrhosis	69	1	96	4
11*	55	M	Metastatic hepatic carcinoma, ? primary site; portal cirrhosis	115	2	83	17
12	84	F	Carcinoma of pancreas, obstructive jaundice	245	2	80	20
13*	65	F	Gall bladder carcinoma, hepatic metastases	276	1	97	3
14*	55	F	Biliary cirrhosis, idiopathic	85	2	88	12
15*	52	F	Pericholangitis, biliary cirrhosis	158	1	100	0
16*	77	F	Biliary cirrhosis, idiopathic	101	2	79	21
17*	52	F	Cholangiolitic cirrhosis	141	2	84	16
18*	80	M	Biliary cirrhosis, extrahepatic obstruction	65	2	85	15
19*	62	F	Choledocholithiasis, obstructive jaundice	74	2	84	16
20*	74	M	Biliary cirrhosis, choledocholithiasis	25	2	76	24
21	73	M	Choledocholithiasis, cholangitis	182	2	74	26
22*	80	M	Stricture of common duct, cholangitis	114	2	86	14
23*	88	M	Choledocholithiasis, obstructive jaundice	304	2	75	25
24*	57	F	Choledocholithiasis, cholangitis	97	2	89	11
25*	55	M	Choledocholithiasis	26	2	95	5
26	45	M	Viral hepatitis	38	1	100	0
27*	58	F	Viral hepatitis	65	1	97	5
28	18	M	Viral hepatitis	21	2	97	3
29	23	F	Viral hepatitis	72	1	100	0
30*	55	M	Fatty nutritional cirrhosis, active	25	2	88	12
31	54	F	Fatty nutritional cirrhosis, active	47	1	96	4
32*	50	F	Fatty nutritional cirrhosis, active	167	1	92	8
33	49	F	Fatty nutritional cirrhosis, active	12	1	97	3
34*	80	F	Fatty nutritional cirrhosis, active	11	1	100	0
35	50	M	Fatty nutritional cirrhosis, active	8	1	100	0
36	65	F	Paget's disease	228	1	99	1
37	74	F	Paget's disease	77	1	98	2
38	73	M	Paget's disease	68	1	99	1
39	54	M	Paget's disease	475	1	99	1
40	61	F	Paget's disease	348	1	99	1
41	69	M	Paget's disease	123	1	99	1
42	62	M	Paget's disease	115	2	99	1
43	72	M	Paget's disease	46	1	98	2
44	70	M	Paget's disease	130	1	95	5
45	71	M	Bronchiogenic carcinoma, bone metastases	68	1	99	1
46	67	M	Prostatic carcinoma, bone metastases	204	1	98	2
47	63	M	Prostatic carcinoma, bone metastases	104	1	97	3
48*	75	M	Prostatic carcinoma, bone metastases	90	2	93	7
49	72	M	Prostatic carcinoma, bone metastases	157	1	100	0
50	77	M	Prostatic carcinoma, bone metastases	257	2	98	2
51	39	F	Breast carcinoma, bone metastases	49	2	95	5
52	52	F	Obesity	11	1	98	2
53	44	F	Non-toxic goiter	7	1	96	4
54	54	M	Myocardial infarction	8	1	100	0
55	35	F	Psychoneurosis	11	1	95	5

* Diagnosis confirmed by anatomical study (biopsy, operation or autopsy).

TABLE I—*Continued*

Case no.	Age	Sex	Diagnosis	Serum alkaline phosphatase (King-Armstrong)	Number of phosphatase peaks	Per cent of activity in	
						alpha-2	alpha-1
56	23	F	Psychoneurosis	12	1	97	3
57	47	M	Psychoneurosis	13	1	97	3
58	15	F	Obesity	12	1	97	3
59	81	M	General arteriosclerosis	9	1	100	0
60	32	F	Hyperthyroidism, in remission	12	1	100	0
61	61	M	Arteriosclerotic heart disease	12	1	100	0
62	30	F	Idiopathic hirsutism	14	1	100	0
63	33	M	Myocardial infarction	12	1	100	0

		Serum alk. phosphatase		Number of cases	Number with 2 peaks	Alpha-1 phosphatase per cent of total (av. ± S.D.)	Number with alpha-1 phosphatase >10% of total
		Range	Average				
Group A	Obstructive and infiltrative biliary disease (Cases 1–25)	25–373	131	25	22	16.2 ± 9.6	21
Group B	Parenchymatous liver disease (Cases 26–35)	8–167	47	10	2	3.5 ± 4.0	1
Group C	Bone disease (Cases 36–51)	46–475	159	16	4	2.2 ± 1.9	0
Group D	Control group (Cases 52–63)	8–14	11	12	0	1.8 ± 1.8	0

agnosis, serum alkaline phosphatase activity, the number of phosphatase peaks, and the proportion of alpha-2 and alpha-1 activity are shown. The cases may be divided into four groups.

Group A (Cases 1 through 25). In this group are included 25 cases of hepatobiliary disease, 13 of which were cases of carcinomatous metastases to the liver, while the remainder included cases of biliary cirrhosis (idiopathic and secondary to extra-hepatic obstruction), choledocholithiasis and cholangitis.

Group B (Cases 26 through 35), consisted of 10 cases of predominantly hepatocellular disease, including four cases of viral hepatitis and six of fatty nutritional cirrhosis in an active phase.

Group C (Cases 36 through 51) was made up of 16 cases of bone disease associated with hyperphosphatasemia and included nine cases of Paget's disease and seven of carcinomatous metastases to bone.

Group D (Cases 52 through 63). This group included 12 subjects with normal serum alkaline phosphatase activity who were hospitalized for a variety of illnesses and were clinically free of liver or bone disease. These were considered normal controls for purposes of this study.

Group A (hepatobiliary disease)

The serum alkaline phosphatase was high in all 25 cases, ranging from 25 to 373 King-Armstrong units. In all but three instances (Cases 10, 13, 15) two distinct phosphatase peaks were present in the electrophoretic pattern, and in 21 of the 22 cases showing both alpha-1 and alpha-2 peaks, the alpha-1 phosphatase exceeded 10 per cent of the total activity. A typical pattern of a case from the group is seen in Figure 1, and also in Figures 2 and 3. The alpha-2 phosphatase was always the predominant component, the maximum value for alpha-1 activity being 44 per cent and the average value, 16.2 per cent. In several cases in this group appreciable activity was found in the beta-globulin zone and in two cases (Cases 7 and 18) a beta-globulin phosphatase peak was found (included in "alpha-2" in the classification). Studies in Case 14 (biliary cirrhosis) were performed at intervals over a 16 month period, during which progressive impairment of liver function occurred (increased gamma globulins, positive Hanger test) and increased fibrosis as revealed by serial liver biopsies; although the data listed in the table show that appreciable alpha-1

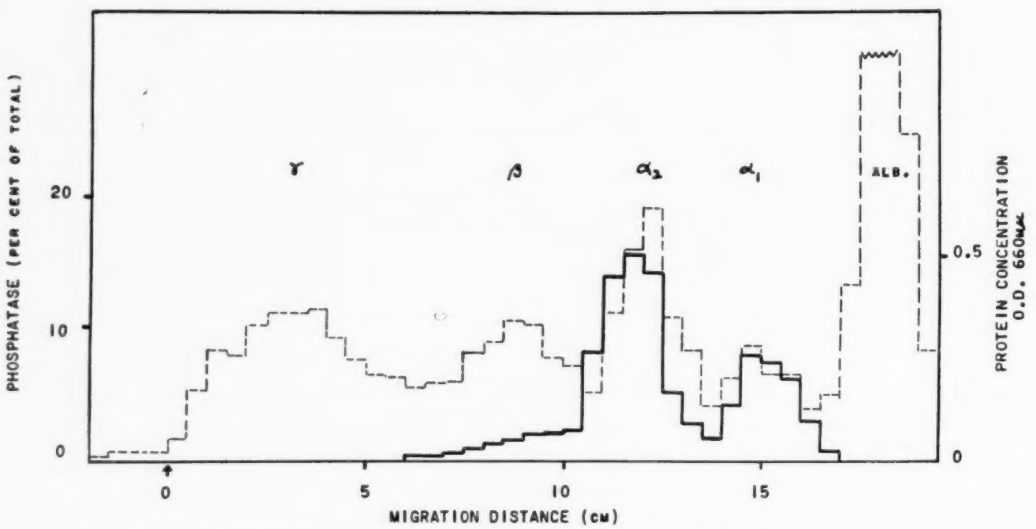

FIG. 1. SERUM ELECTROPHORETIC PATTERN, CHOLANGIOLITIC HEPATITIS (CASE 17)

In this and the remaining figures, the abscissa indicates the distance from the line of application of the sample, indicated by the arrow. The anodal direction is to the right. Phosphatase activity (solid lines) and protein concentrations (broken lines) are indicated by the height of adjacent vertical bars, of which only the noncontiguous portions are delineated. Each bar represents a single starch segment. Phosphatase is expressed as a fraction of the total activity in the pattern, protein concentration as optical density of starch filtrates as determined by the modified Folin method (10).

The serum specimen was obtained two months after surgical exploration which confirmed the diagnosis of cholangiolitic cirrhosis. The serum phosphatase was 113 King-Armstrong units. Two distinct phosphatase peaks are seen, the alpha-1 zone accounting for 29 per cent of the total. In the serum specimens obtained preoperatively, one month after onset of the disease, the alpha-1 phosphatase peak contributed 16 per cent of the total, as indicated in Table I.

activity was present in the first study, in two later studies no distinct alpha-1 peak was found, and only 5 to 8 per cent of the activity was in this region.

There seemed to be no correlation between the presence or degree of bilirubinemia and the value for the fraction of the total phosphatase activity in alpha-1, nor was this value related to the value of total alkaline phosphatase of the serum. Review of the clinical and laboratory data of the four cases with less than 10 per cent alpha-1 activity failed to show differences from the 21 cases in which alpha-1 activity exceeded 10 per cent.

Group B (hepatocellular disease)

Of the 10 cases, three had normal serum phosphatase values. In two of the 10, the electrophoretic phosphatase pattern revealed both alpha-1 and alpha-2 peaks, alpha-1 being 3 and 12 per cent. The average value for alpha-1 phosphatase in this group was 3.5 per cent. The serum protein pattern was abnormal in most of these cases, gamma globulins being increased and albumin decreased. In some patterns the phosphatase activity in alpha-2 had a broader base and the peak was not as prominent nor as sharp as in Groups A and C, and in two cases (31 and 33) a distinct peak of activity was present in the beta region.

Group C (bone disease)

Four of the 16 cases of hyperphosphatasemic bone disease had phosphatase patterns characterized by an alpha-1 as well as an alpha-2 peak, but the proportion of the total in the alpha-1 zone was small (average, 2.2 per cent; maximum, 7 per cent). The predominant alpha-2 phosphatase zone tended to have a sharp, distinct peak, with relatively less of a beta trail than was seen in the other groups, and the alpha-2 zone of activity was consequently more symmetrical. The protein pat-

tern revealed no consistent abnormalities although in several cases of Paget's disease the alpha-2 globulin fraction was more prominent than normal. An example of the pattern in a case of Paget's disease is shown in Figure 4.

Group D (control group)

The electrophoretic phosphatase patterns in the "control" cases were qualitatively similar to those in the bone disease cases. None of these cases showed a distinct alpha-1 phosphatase peak although a small fraction of the total activity (range, 2 to 5 percent; average, 1.8 per cent) was present in the alpha-1 globulin zone. An appreciable fraction of the "alpha-2" activity was in fact present as a trail in the beta globulins; this made the alpha-2 peaks less sharp and distinct than the corresponding peak in the bone cases. A normal pattern is shown in the lower panel of Figure 5. A possible explanation of the trailing might be that trailing or adsorption of a quantity of the alpha-2 phosphatase occurred in all cases, but a

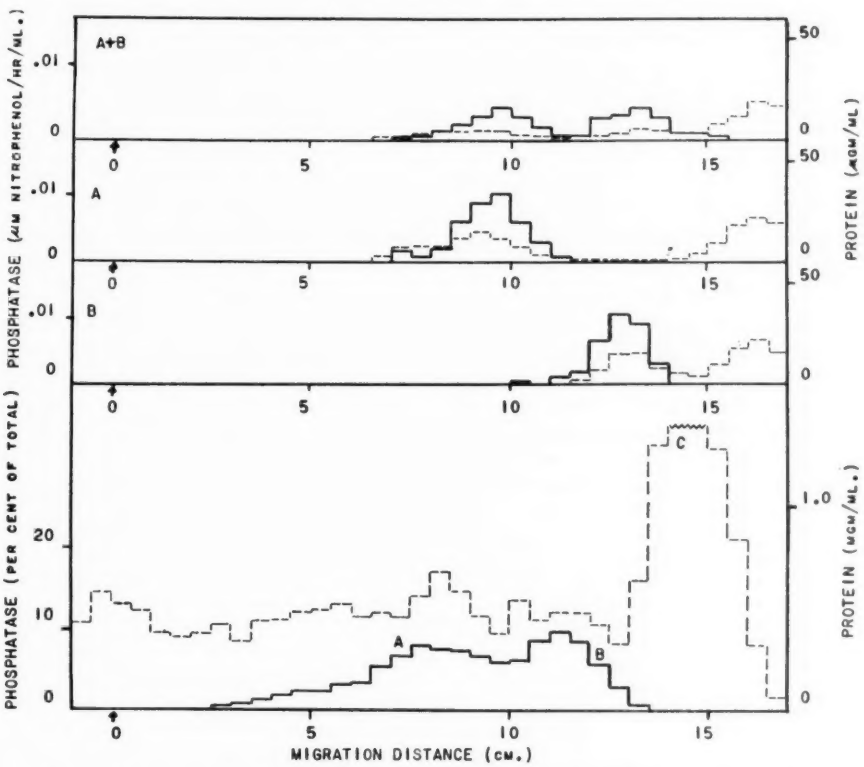

FIG. 2. ELECTROPHORETIC PATTERNS OF SERUM AND OF FRACTIONS OF THE ALPHA-2 AND ALPHA-1 GLOBULINS, CASE 5 (BOWEL CARCINOMA METASTATIC TO LIVER)

The electrophoretic pattern of the serum performed on a preparative scale is shown in the lower panel. Alpha-1 phosphatase is 42 per cent of the total. Samples of eluates of segments A (alpha-2) and B (alpha-1) and a mixture of the two (A plus B) were then run separately in small starch blocks simultaneously, a quantity of albumin (segment C eluate) having been added to each sample as a reference standard for mobility; the patterns shown in the upper panels, labeled A, B and A + B, correspond to the respective samples. In the mixture of A + B, 44 per cent of the phosphatase was derived from alpha-1 (B) and 56 per cent from alpha-2 (A); in the pattern of A + B, 53 per cent of the activity was in the alpha-1 zone. The characteristic mobilities of the two phosphatases observed in the electrophoresis of the individual fractions are preserved in the pattern of the mixture, and resemble those in the serum pattern.

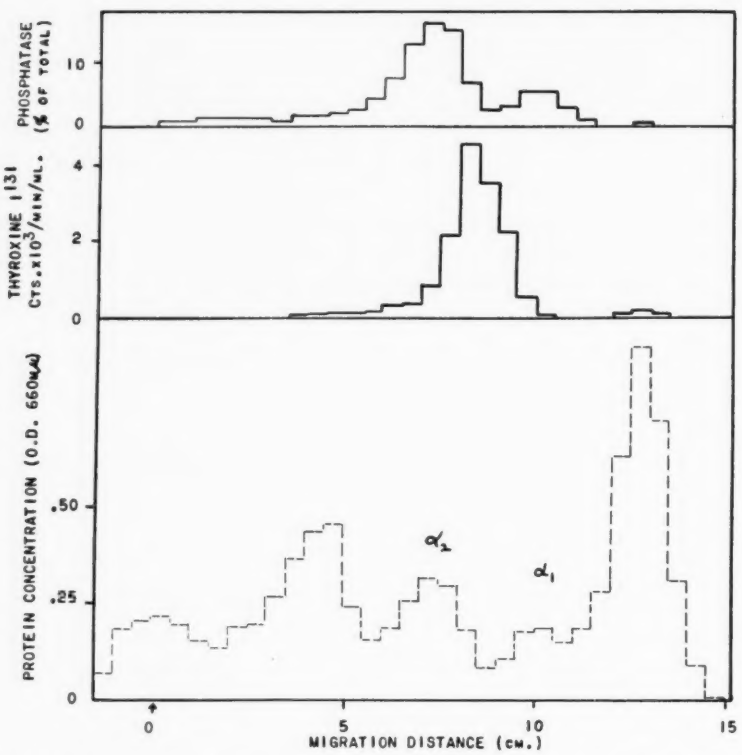

FIG. 3. ELECTROPHORETIC PATTERN OF SERUM, CASE 9 (CARCINOMA OF GALL BLADDER WITH HEPATIC METASTASES)

One-tenth ml. of I^{131}-labeled thyroxine (0.5 μg.) was added to a mixture of 0.5 ml. serum, and 0.5 ml. barbital buffer; 0.7 ml. of the resulting solution was used for electrophoresis. The lower panel shows the serum protein pattern, and the upper panel the alkaline phosphatase distributed in two well-resolved zones, the alpha-1 zone accounting for 20 per cent of the total. The middle panel shows the distribution of radioactive iodine in the pattern, with a large peak in the "inter-alpha" region and a small peak in albumin. The mobility of the thyroxine-binding protein is greater than the alpha-2 phosphatase and less than the alpha-1.

smaller proportion of the total activity was in the trail when the total serum alkaline phosphatase was high, as it was in the Group C cases.

The lower part of Table I summarizes the results in the four different groups studied. In group A, 84 per cent of the cases had more than 10 per cent of the total phosphatase activity in the alpha-1 zone, while only one of the 38 cases in the remaining three groups showed an alpha-1 zone exceeding this value.

The difference in the mobilities of the alpha-2 and alpha-1 phosphatase activities found in the serum electrophoretic patterns of most of the patients in Group A was apparent in two other types of experiments.

a). Hyperphosphatasemic sera from cases of bone disease showing only alpha-2 phosphatase in the electrophoretic patterns were mixed with sera from cases of hepatic cancer in which both alpha-2 and alpha-1 phosphatase zones were present. Electrophoresis of such mixtures (three cases) resulted in a phosphatase pattern suggestive of an additive effect, the pattern of the mixture being similar to that obtained by adding the values from the patterns of each serum alone. The resolution of the two peaks in the mixture was generally

inferior to that in the original serum containing the two activities, but alpha-1 phosphatase activity was evident in the mixture pattern.

b). In several experiments such as that shown in Figure 2, preparative scale electrophoresis was done with a serum sample containing alpha-2 and alpha-1 phosphatase peaks, and aliquots of the eluates from each phosphatase zone were then subjected to electrophoresis separately and as a mixture. The mobilities of the fractions separated from the serum and run independently differed distinctly from each other, and electrophoresis of the mixture of fractions effected complete resolution with preservation of the individual mobilities, which were similar to those observed in the original serum pattern; the proportion of each activity in the resolved mixture was comparable to the composition of the prepared mixture.

These results suggested that the occurrence of the two phosphatase zones in some sera was probably indicative of the presence of more than one alkaline phosphatase, rather than of a single phosphatase distributed into two electrophoretically distinct forms as a result of protein-protein interactions. Other experiments also failed to show such interaction. Thus, there seemed to be no relationship between the prominence of the alpha-1 globulin zone in the serum electrophoretic pattern and the magnitude of the alpha-1 phosphatase activity. In another study of possible interactions a serum sample from a case of bone disease (Case 47) was subjected to electrophoresis in a preparative block; the pattern showed almost all the alkaline phosphatase activity in the alpha-2 zone, and the alpha-1 globulins, although well resolved, had little activity. Electrophoresis was then performed with eluates of single segments of the alpha-2 zone, of the alpha-1 zone, and of a mixture prepared from both zones in such proportions that the concentration of alpha-1 globulin (phosphatase-poor) in the mixture was 2.5 times the concentration of the alpha-2 protein; the resulting patterns showed that the proteins were well resolved according to their characteristic mobilities, and the phosphatase activity was confined to the alpha-2 globulins in the patterns of the mixture as well as of the separate fractions. This again suggested that interaction of enzyme with inert protein carriers was not a major mechanism of the occurrence of the two electrophoretically distinct phosphatase activities.

The mobilities of the two phosphatases, corresponding closely but not always precisely with the peaks of alpha-2 and alpha-1 globulins, were studied further by comparison with the mobility of the "thyroxine-binding protein." A small quantity of I^{131} labeled thyroxine was added to serum before electrophoresis on a starch block and the distribution of radioactivity was compared with that of phosphatase. Three such experiments

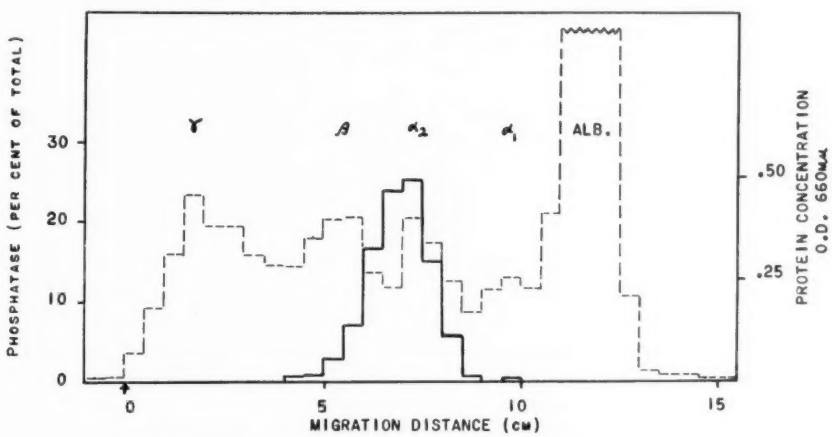

FIG. 4. SERUM ELECTROPHORETIC PATTERN, PAGET'S DISEASE (CASE 42)

Most of the phosphatase activity is in a single zone in the alpha-2 globulins, and only 1 per cent of the activity is in the alpha-1 globulin zone.

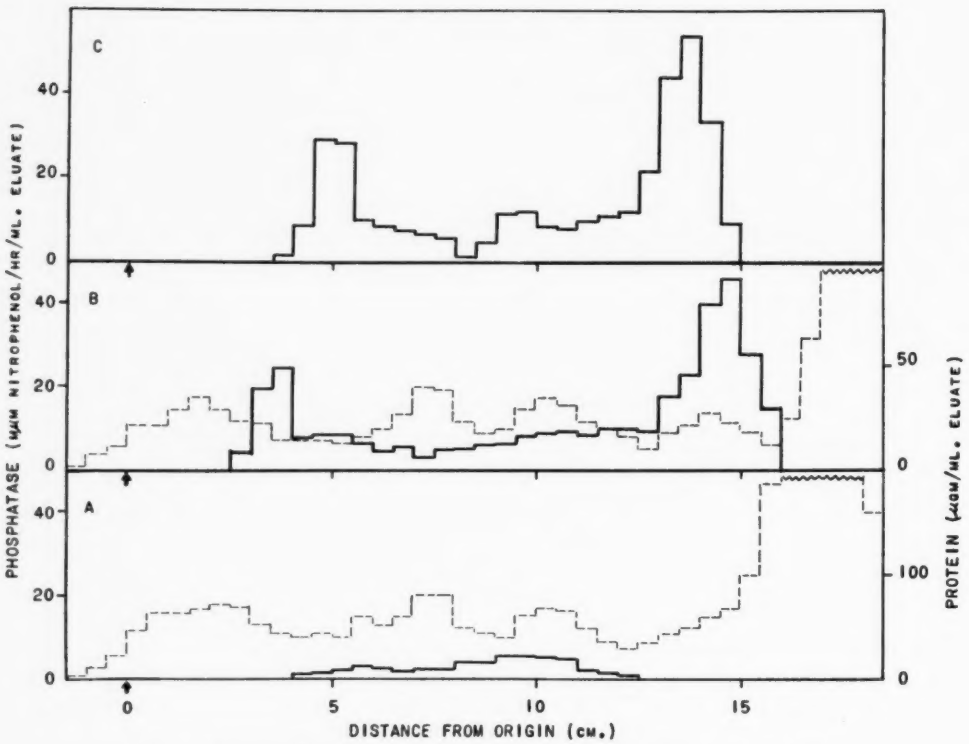

FIG. 5. ELECTROPHORETIC PATTERN OF BILE AND OF NORMAL SERUM (CASE 62)

The specimen of bile (alkaline phosphatase 164 King-Armstrong units) was obtained from a T-tube in the common duct of a patient eight days after operation for choledocholithiasis. The serum specimen had 14 K-A units alkaline phosphatase. The patterns were obtained by simultaneous electrophoresis in three parallel blocks.

Lower panel: serum electrophoretic pattern. The alpha-2 phosphatase is predominant, with a long beta trail.

Upper panel: electrophoretic phosphatase pattern of bile. The predominant activity corresponds to alpha-1 globulin in mobility, with smaller zones of activity at beta and alpha-2. The protein concentration was very small and is not indicated.

Middle panel: electrophoretic pattern of mixture of equal parts of bile and serum. Most of the phosphatase activity is contributed by the bile and the largest phosphatase zone corresponds to the alpha-1 globulins. There was considerable loss of activity in the electrophoresis of bile alone (C) and smaller losses during electrophoresis of the bile-serum mixture.

The phosphatase scale is the same in all three charts; the protein scale in the middle panel is twice that in the lower.

yielded results similar to the patterns shown in Figure 3. The thyroxine-binding protein, as judged by the position of the predominant band of radioactivity, was found to be in the "inter-alpha" postion of the electrophoretogram in confirmation of the findings of other (16, 17), and the alpha-2 and alpha-1 phosphatases were found on the cathodic and anodic sides, respectively, of the thyroxine-binding protein.

Studies of the pH optimum and of the effects of inhibitors upon enzyme activity have thus far failed to show differences between alpha-2 and alpha-1 phosphatase. In four experiments in which pH-phosphatase activity curves of eluates from the alpha-2 and alpha-1 segments of starch blocks were compared, no differences in the shape of the curves nor in the pH optimum of the two phosphatases were found (Figure 6). The value

of the pH optimum was approximately 9.8 in experiments using 2-methyl-2-amino-propanol as buffer and *p*-nitrophenylphosphate as substrate. Table II shows the effects of adding cyanide and fluoride to solutions of separated alpha-2 and alpha-1 phosphatases; both were almost equally inhibited by cyanide and insensitive to fluoride.

The effect of adding albumin to phosphatase fractions was studied since it has been reported that the addition of albumin to serum decreases serum alkaline phosphatase activity (18). Phosphatase-free albumin solutions, derived from the albumin zone of preparative starch block electrophoresis of serum, were added to solutions of similarly isolated alpha-2 and alpha-1 phosphatase fractions so that 80 per cent of the total protein in the mixtures was albumin, and the enzymatic activity of the mixtures was compared with that of the phosphatase fractions free of albumin. No inhibitory effect of albumin was observed, nor did gamma globulin have any effect in analogous experiments. Mixtures of alpha-2 and alpha-1 phosphatase fractions had enzymatic activity equal to the sum of the components separately. In one (Case 10) of the three cases in Group A in which little alpha-1 phosphatase was present, no effect on enzyme activity was observed when alpha-1 globulin obtained by zone electrophoresis of this serum was mixed with alpha-2 phosphatase from this case. This suggested that the absence of alpha-1 phosphatase in this case was not attributable to a phosphatase inhibitor in the alpha-1 globulins.

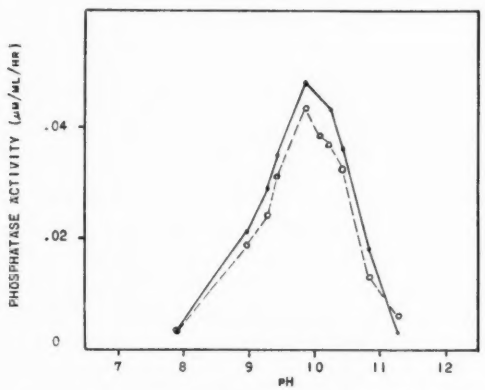

FIG. 6. PHOSPHATASE ACTIVITY-pH CURVES OF ALPHA-2 AND ALPHA-1 PHOSPHATASES

Eluates of single segments from the alpha-2 and alpha-1 zones of the serum electrophoresis in Case 21 were used; as in Figure 2, the segments were chosen from the cathodal limb of alpha-2 and the anodal limb of alpha-1 in order to minimize possible admixture due to incomplete electrophoretic resolution. Aliquots of each of the enzyme solutions were added to a series of tubes containing substrate-buffer mixtures of varying pH. The pH recorded is that of the mixture of enzyme with buffered substrate solution as determined in a parallel set of unincubated tubes. The pH optimum is approximately 9.8 for each phosphatase.

Unbroken line represents alpha-2; broken line, alpha-1.

TABLE II

Effect of cyanide and fluoride on alpha-2 and alpha-1 phosphatase *

Substance added	Final concentration	Phosphatase activity (% of control) alpha-2	alpha-1
None		100	100
Sodium cyanide	10^{-4} M	94	100
Sodium cyanide	10^{-3} M	59	64
Sodium cyanide	10^{-2} M	5	10
Sodium fluoride	10^{-4} M	97	100
Sodium fluoride	10^{-3} M	100	100
Sodium fluoride	10^{-2} M	97	100

* The solutions of alpha-2 and alpha-1 phosphatase, each containing 40 μg. protein per ml. and nearly equal phosphatase activity, were obtained from starch block electrophoresis of Case 5.

In one of the cases of prostatic cancer with bone metastases (Case 48) the serum acid phosphatase, as well as the serum alkaline phosphatase, was very high and the distribution of acid phosphatase activity in the starch filtrates was determined with the result shown in Figure 7. The acid phosphatase was found in a fairly discrete zone of greater mobility than the alpha-2 alkaline phosphatase and alpha-2 globulins but of lesser mobility than the alpha-1 phosphatase or alpha-1 globulin. The pattern in this case seemed to indicate clearly the separate nature of serum alkaline and acid phosphatases.

Bile phosphatase

Nine specimens of bile were studied; they were obtained either from the gall bladder at autopsy or from cholecystostomy and choledochostomy tubes of postoperative cases of biliary disease. The values of alkaline phosphatase in these samples ranged between 58 and 185 King-Armstrong units and averaged 127. The most satisfactory starch electrophoretic patterns were obtained with bile which had not been frozen and which was

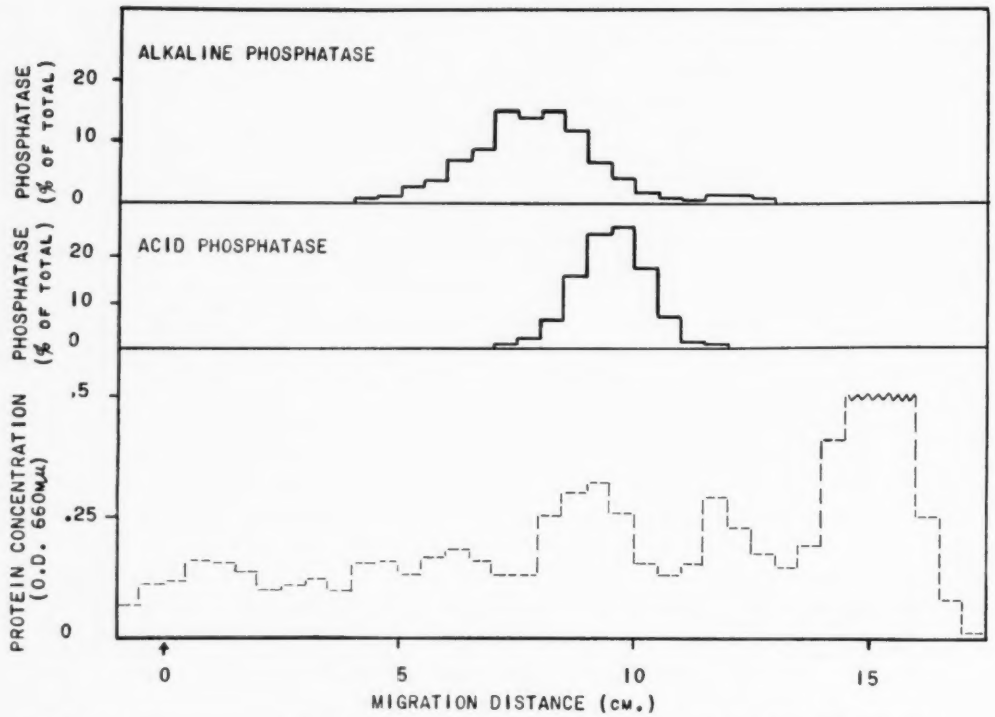

FIG. 7. ELECTROPHORESIS OF SERUM, CASE 48 (PROSTATIC CARCINOMA WITH SKELETAL METASTASES)

The serum acid phosphatase was over 2,000 King-Armstrong units. Lower panel, protein pattern; upper panel, alkaline phosphatase; middle panel, acid phosphatase. A small alpha-1 zone of alkaline phosphatase is present. The acid phosphatase peak lies between the two alkaline phosphatase zones.

filtered before use. The electrophoretic patterns of bile alone (six cases) were somewhat variable; a slow component of mobility similar to the beta globulins was seen in some, and in all cases a phosphatase zone of mobility approximating that of serum alpha-1 globulin was found as well as a phosphatase of alpha-2 globulin mobility. There was little protein in most of the bile samples and estimation of comparative mobilities was made by comparing the zones of enzymatic activity in the bile patterns with the protein bands of serum specimens run simultaneously in a parallel starch block. When bile was added to serum containing predominantly alpha-2 phosphatase, the electrophoretic pattern of the mixture resembled, in an exaggerated way, the alpha-2 plus alpha-1 pattern of most Group A cases. An example of this is seen in Figure 5, in which bile of high phosphatase activity was added to normal serum (Case 62); the predominant phosphatase in the mixture, as well as in bile alone, had the mobility of alpha-1 globulins, while the serum alone had no alpha-1 activity. The bile and bile-serum mixture also contained an appreciable beta zone activity, and there was some alpha-2 activity.

Phosphatase recovery and reproducibility of pattern

Between 51 and 68 (average, 60) per cent of the phosphatase in the serum sample subjected to electrophoresis was recovered in the starch filtrates (eight experiments). These estimates were subject to considerable error since they involved comparison of the phosphatase activity of the serum with the sum of the activities of 30 to 40 starch segment eluates. In the calculation, it was assumed that all the phosphatase in the starch segment was distributed in a volume equivalent

to the volume of water used for elution plus the water content of the damp segment (determined by weighing several segments before and after desiccation). These figures do indicate, however, that very large losses of activity probably did not occur during the electrophoresis. Satisfactory recoveries were also obtained when isolated eluates of the two phosphatases were re-run on blocks. Large losses (80 per cent) were encountered when bile was subjected to electrophoresis; addition of serum to the bile sample seemed to stabilize the activity, and losses were smaller.

The qualitative and quantitative aspects of the phosphatase patterns of a particular serum were reasonably reproducible. The difference between values for the proportion of alpha-1 phosphatase in duplicate runs was seldom greater than 20 per cent of the larger value.

Since the serum mucoprotein concentration is abnormally high in hepatic cancer and low in hepatocellular disease (19), since certain alkaline phosphatases contain carbohydrate (20), and since the fraction of phosphatase in alpha-1 was larger in hepatic cancer then in hepatitis, serum protein-bound hexose determinations were made in 40 cases in which the relative proportions of alpha-2 and alpha-1 phosphatase had been measured. There seemed to be no relationship between the serum glycoprotein concentration and the occurrence or magnitude of alpha-1 phosphatase activity.

DISCUSSION

The localization of serum alkaline phosphatase activity in the alpha globulin zone agrees both with the observation that phosphatase activity occurs in the alpha globulin prepared by alcohol fractionation of plasma (21) and also with results of paper electrophoretic studies of serum phosphatase in man (22–24). Eisfeld and Koch (22), using paper electrophoresis, reported that normally the serum alkaline phosphatase activity was in the alpha-2 globulin zone, while in hyperphosphatasemic states phosphatase activity was also present in the alpha-1 globulins; apparently they observed no difference between the phosphatase patterns in bone disease and in biliary obstruction. Baker and Pellegrino (23) also used paper electrophoresis and found alkaline phosphatase activity in the alpha-2 globulins, and in some cases observed an additional zone of activity in the beta globulins.

The present studies, in which starch block electrophoresis was used, yielded results which are not in complete agreement with those reported in the paper electrophoretic studies. Although the alpha-2 phosphatase was the predominant component found in all cases in the present study, an additional phosphatase in the alpha-1 globulin zone contributed an appreciable fraction of the total alkaline phosphatase activity in most hyperphosphatasemic cases of infiltrative and obstructive hepatobiliary disease. This contrasted with the normal cases and with the bone disease cases in which alpha-1 phosphatase activity was either absent or present as only a small fraction of the total (less than 10 per cent). It is concluded that the occurrence of an appreciable fraction of the serum phosphatase activity in the alpha-1 fraction is related to the nature of the clinical disorder associated with the increase in serum phosphatase activity rather than to the degree of the increase. Because of the qualitative similarity of the normal patterns and those of bone disease, it is believed that the pattern in which more than ten per cent of the phosphatase is in the alpha-1 fraction is abnormal. The occurrence of the abnormal alpha-1 phosphatase accounts for relatively little of the hyperphosphatasemia of biliary disease, the alpha-1 phosphatase being found to have an average value of only 16 per cent of the total serum alkaline phosphatase.

The results of the studies demonstrating the independence of the mobilities in mixtures of isolated fractions of alpha-2 and alpha-1 phosphatase and the failure to find modification of enzyme activity or mobility by addition of various protein fractions lead to the suggestion that the alpha-2 and alpha-1 phosphatase activities represent distinct enzymes, although thus far the difference in electrophoretic mobility has provided the only distinguishing feature.

It is of interest that in a recent study of serum fractionated by a column chromatographic procedure, evidence was presented suggesting the occurrence of two alkaline phosphatases in the serum of a patient with carcinoma metastatic to bone and liver (25). It has also been reported that after experimental biliary obstruction in the rat, an alpha-1 phosphatase is demonstrable by paper

electrophoresis of the serum, which normally contains an alpha-2 and a beta zone alkaline phosphatase (26).

The electrophoretic patterns of bile and of bile-serum mixtures showed that a phosphatase of mobility approximating alpha-1 globulin was present in bile and in some instances it was the major component. Although similarity of electrophoretic mobility is not proof of the identity of the alpha-1 phosphatase of bile and of serum, these observations are at least consistent with the idea that in hyperphosphatasemic biliary disease the serum may contain an appreciable quantity of an alkaline phosphatase which normally represents only a very small fraction of the total phosphatase of serum but which may be the predominant phosphatase in bile. The alpha-1 phosphatase of bile could represent a serum phosphatase (? alpha-2) which during the course of biliary excretion has undergone some transformation resulting in changed electrophoretic properties, or it might be a secretory product of hepatic origin excreted in bile. Regardless of the origin of the alpha-1 biliary phosphatase, the presence of an appreciable fraction of serum phosphatase of similar mobility is compatible with the view that the liver may in some cases contribute to the serum phosphatase activity. Another possible explanation might be that the alpha-2 and alpha-1 phosphatases are normally introduced into the circulation from the tissues of origin, but that the biliary excretion of alpha-1 phosphatase is more rapid or complete than the excretion of alpha-2; the lack of a prominent alpha-1 component in most cases of hepatocellular disease would make the latter explanation less likely. Although the serum alpha-1 phosphatase

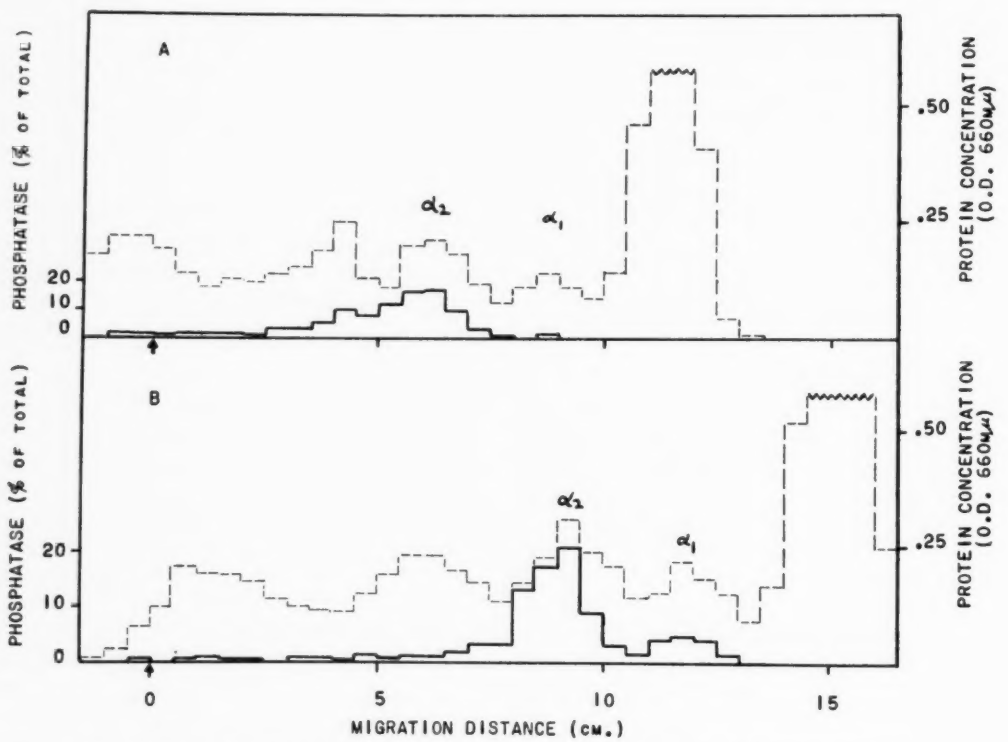

FIG. 8. SERUM ELECTROPHORETIC PATTERNS IN TWO CASES OF METASTATIC BREAST CARCINOMA WITH HYPERPHOSPHATASEMIA

The electrophoreses were not simultaneously performed, and the total traverse in both cases is not the same.
A. Case 51, bony metastases only: five per cent of the activity is in the alpha-1 region.
B. Case 6, metastases to bone, liver and other organs: 19 per cent of the activity is in the alpha-1 region.

was detected most regularly in cases of hepatic cancer, it would seem unlikely that it is of neoplastic origin, both because it was present in cases of cholangitis and noncarcinomatous biliary obstruction and also because it was absent in cases of disseminated cancer of the prostate and breast in which there were no hepatic metastases.

Although there was some overlap in phosphatase patterns between cases of hepatocellular disease and of obstructive or infiltrative liver disease, and although a few of the latter cases had patterns indistinguishable from those of bone cases, the general tendency toward the occurrence of a prominent alpha-1 phosphatase zone in obstructive and infiltrative biliary disease and its absence in the other cases studied suggests a possible clinical usefulness for this method of study in some cases of increased serum alkaline phosphatase of obscure nature. The method might be particularly useful in cases where hepatic metastatic lesions were suspected. Figure 8 shows the serum electrophoretic patterns in two cases of carcinoma of the breast with high serum alkaline phosphatase; in the case in which skeletal metastases were radiologically demonstrable and there was no evidence of hepatic metastases (upper panel) the pattern revealed a prominent alpha-2 phosphatase with some trail in the beta zone, and only a small alpha-1 phosphatase peak (5 per cent of the total), while in the case in which metastases had occurred in liver as well as bone and other tissues, a prominent alpha-1 zone was noted. Patterns obtained in two cases of sarcoidosis and in three cases of disseminated tuberculosis, in all of which the serum alkaline phosphatase activity was moderately increased, revealed appreciable alpha-1 as well as alpha-2 zones of activity; biopsy evidence was not available and, therefore, the suspicion that these patterns suggested hepatic involvement is not proven.

Evaluation of the possible clinical usefulness of this approach would be facilitated by the development of simpler methods for demonstrating abnormally high proportions of alpha-1 phosphatase.

SUMMARY

1. Electrophoresis on starch blocks of sera of increased alkaline phosphatase activity indicated the presence in some cases of two zones of alkaline phosphatase activity with mobilities corresponding to alpha-2 and alpha-1 globulins. The alpha-2 fraction was the larger in all cases. In bone disease the average value for the proportion of alpha-1 phosphatase was 2.2 per cent, while the corresponding value in cases of hepatobiliary infiltrative and obstructive disease was 16.2 per cent. Normal sera and those from cases of hepatocellular disease showed only a small percentage of the alkaline phosphatase activity in the alpha-1 zone, averaging 1.8 and 3.5 per cent, respectively.

2. Alpha-2 and alpha-1 phosphatases were similar in pH optimum, cyanide sensitivity and fluoride insensitivity. The difference in electrophoretic mobilities was preserved in mixtures of the two phosphatases.

3. An appreciable fraction of the alkaline phosphatase of bile had mobility similar to the alpha-1 globulins.

4. Zone electrophoretic study may be clinically useful in some cases of obscure hyperphosphatasemia.

ACKNOWLEDGMENTS

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STUDIES ON BILIRUBIN SULFATE AND OTHER NONGLUCURONIDE CONJUGATES OF BILIRUBIN * †

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As a result of the important observations of Billing, Cole and Lathe (2, 3), Schmid (4) and Talafant (5), it is now clear that "direct-reacting" bilirubin is a polar and water soluble conjugate of bilirubin. These investigators have provided evidence that this conjugate is a diglucuronide formed primarily in the liver, and recently it has been shown that the glucuronic acid is attached at the two carboxyl groups of the bilirubin molecule (6). Since the hepatic conjugation of alcoholic and phenolic compounds, such as corticosteroids and estrogens, involves the formation of sulfate as well as glucuronide derivatives, it seemed reasonable that an ethereal sulfate derivative of bilirubin at its hydroxyl groups might also occur. Such a derivative would be water soluble, but in contrast to the alkali labile glucuronide ester, the ethereal sulfate would be alkali stable. Previous observations by Billing and associates (3) that 5 to 40 per cent of human bile pigments were alkali stable gave additional support for this hypothesis.

The present study provides evidence for the physiologic occurrence of bilirubin sulfate as well as other, as yet unidentified, nonglucuronide conjugates of bilirubin in the bile of animals and man.

MATERIALS AND METHODS

Experiments with radioactive sulfate. Polyethylene catheters were inserted into the bile ducts of rats, after which the animals were injected subcutaneously and intraperitoneally with 1 to 2 μc. of inorganic $S^{35}O_4$ in normal saline. Bile was collected while the animals were in restraining cages. In cats, the bile was collected from the gall bladder and in man from T-tube drainages after the injection of labeled sulfate.

For diazotization bile was first brought to pH 4 to 5. Then diazotization was carried out in the usual manner (3) with sulfanilic acid, *p*-aminobenzoate or aniline.

Purification procedures and chromatography. The diazotized pigments were washed with heptane-butanol and chloroform (7) and then extracted into *n*-butanol. The solvent was brought to dryness by vacuum distillation in a flash evaporator and redissolved in water. For further purification, the pigments were added to a suspension of $Zn(OH)_2$ gel at pH 6. The gel was washed as previously described (1) and then dissolved in 0.5 N HCl. The pigments were again extracted into *n*-butanol, the solvent removed by vacuum distillation and prepared for chromatography by dissolving in 1 N acetic acid.

Ascending paper chromatography was carried out on Whatman No. 1 paper using three solvent systems: *n*-propionic acid-methyl ethyl ketone-water (25:75:30) (4); *n*-butanol-glacial acetic acid (4:1); pyridine-ethyl acetate-water (1:2:1). Pigments were eluted from paper with 0.05 N HCl and their absorbency at 560 mμ determined in a Beckman DU spectrophotometer. The molecular extinction coefficient for azobilirubin under these conditions was 11.5.

Enzymatic hydrolysis with bacterial β-glucuronidase (obtained from Sigma Chemical Company, St. Louis, Mo.) was carried out at 37° C. for 24 hours at pH 6.2 using acetate buffer at a final concentration of 0.1 M. Fifty mg. of enzyme was added initially and again 12 to 16 hours later. The sulfatase preparation was a commercial arylsulfatase known as Mylase-P® (Wallerstein Laboratories, New York).

Reactions with hydroxylamine were carried out as described by Schachter (6). Methylation was carried out by exposing the pigments on paper to an atmosphere of diazomethane. Diazomethane was prepared from N-methyl-N-nitroso-*p*-toluene sulfonamide (8). The latter was obtained as Diazald® from the Aldrich Chemical Company, Milwaukee, Wis.

Sulfate determination. The bile azopigments were washed and purified as above and chromatographed in the pyridine system. After elution, the amount of azopigment was determined spectrophotometrically. Two 1 ml. aliquots of the pigments were transferred to test tubes to which was added 1 ml. of 20 per cent trichloracetic acid. One tube was heated at 100° C. for 90 minutes, while the other was kept at 0° C. as a control. Then 5 ml. of 1 per cent recrystallized benzidine was added to each tube and the sulfate measured by the method of Dodgson and Spencer (9). The sulfate liberated by hydrolysis was determined from a standard curve which had been prepared using potassium sulfate. Hydrocholic acid was used

[1] This investigation was supported in part by Research Grant A-1392 from the National Institutes of Health.

† A preliminary communication of this work has appeared previously (1).

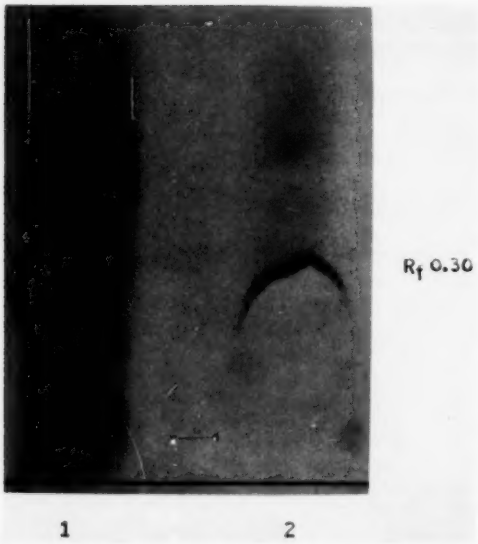

FIG. 1. CHROMATOGRAPHY OF RAT BILE AFTER INJECTION OF RADIOACTIVE SULFATE

Chromatographic system was the propionic acid system of Schmid (4). Column 1 indicates the results of the bile after diazotization and extraction into butanol. Column 2 is the same bile but after purification with $Zn(OH)_2$ gel.

initially in the hydrolysis but was abandoned because it interfered with the sulfate method and gave erratic results.

Preparation of synthetic "bilirubin sulfate." 1. Reaction with Liebermann-Burchard reagent. Bilirubin was dissolved in chloroform and then reacted with the Liebermann-Burchard reagent (10). Usually inorganic $S^{35}O_4$ was also added. After one minute, the reaction mixture was added to water and the lower chloroform layer removed. The yellow-green aqueous solution was then washed three times with chloroform and diazotized with sulfanilic acid. The azopigments were extracted into *n*-butanol and the latter removed by vacuum distillation as above. The dried pigments were then dissolved in water or 1 N acetic acid.

2. Chlorosulfonic acid method. To a solution of 10 mg. of bilirubin in 10 ml. of dry pyridine at 0° C. was added dropwise 1 ml. of redistilled chlorosulfonic acid (11). Constant stirring with a magnetic stirrer was maintained during the addition of the acid and continued for 30 minutes thereafter. After standing overnight at 0° C., ice was added to dilute the chlorosulfonic acid. The pH was adjusted to pH 4 to 5 and diazotized sulfanilic acid added. Extraction into butanol was carried out as above.

Other procedures. Stability of pigments to alkali was carried out by neutralizing the solutions of azopigment and then adding NaOH to give a final concentration of 0.1 N. After standing at room temperature for 10 minutes, the solution was acidified and extracted into *n*-butanol.

Glucuronic acid was measured by the naphthoresorcinol method as described by Fishman and Green (12).

RESULTS

Demonstration of S^{35}-labeled azopigments in bile

In the initial studies carried out in rats with biliary fistulas, the injection of S^{35}-labeled sulfate resulted in the excretion of numerous radioactive products in bile as shown by paper chromatography and autoradiography (Figure 1, Chromatogram 1). After purification by extraction procedures, followed by adsorption and elution from $Zn(OH)_2$ gel, one major radioactive metabolite remained which migrated with the conjugated azopigment of bile (Figure 1, Chromatogram 2). When this band was eluted and rechromatographed in any of three solvent systems, the radioactivity still remained with the azopigment band (Figure 2). Acid hydrolysis of this eluted

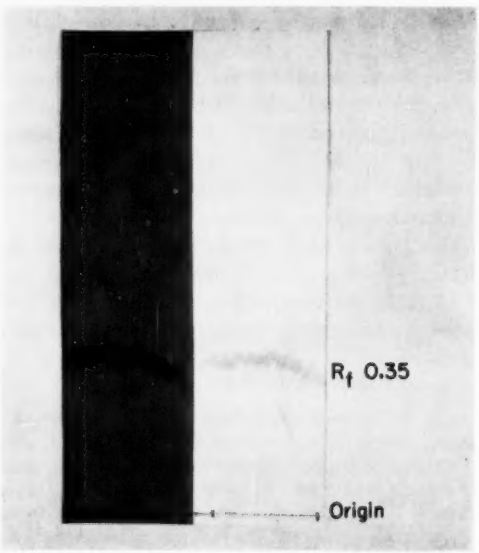

FIG. 2. RECHROMATOGRAPHY AND AUTORADIOGRAPHY OF RAT BILE AZOPIGMENT AFTER INJECTION OF RADIOACTIVE SULFATE

This conjugated azopigment was eluted from the propionic acid system and rechromatographed in the pyridine system. The azopigment is pictured on the right and the corresponding autoradiograph is shown at the left. It will be noted that the radioactivity directly coincides with pigment zone.

TABLE I

Analysis of human T-tube bile azopigments

Patient no.	Per cent β-glucuronidase resistant*	Per cent containing sulfate†	Per cent alkali stable‡
B-1	16.8	7.0	6.8
B-2	16.7	9.4	11.7
B-3	19.9	13.6	14.9
B-4	20.0	11.1	12.2
B-6	16.7	7.1	6.8
B-8	28.8	17.5	16.4
B-10	30.4	15.0	20.2
B-11	28.0	8.0	9.4
B-14	34.6 (28.6)	22.6	24.6
B-15	27.4 (26.0)	19.6	21.9
B-16	20.9 (20.0)	20.1	20.0
Mean	23.7	13.7	15.0
S. D.	±6.4	±5.6	±6.2

* Azopigments were treated as described in text and exposed to β-glucuronidase for 24 hours at 37° C. Parentheses indicate values of β-glucuronidase resistant fraction, where azopigments were first chromatographed and eluted prior to enzyme treatment. These values have not been included in the calculation of the mean or standard deviation.

† Analyses for acid hydrolyzable sulfate were performed on β-glucuronidase resistant fraction and expressed as per cent of total pigment (micromoles of inorganic sulfate liberated per micromoles of total azopigment).

‡ Expressed as per cent of conjugated azopigment remaining after exposure to a final concentration of 0.1 N NaOH for 10 minutes at 25° C.

labeled pigment in 1 N HCl at 100° for 90 minutes resulted in the liberation of radioactive inorganic sulfate, as demonstrated by chromatography. Taurine was not detected as a product of this hydrolysis at any time. Similar results have been obtained in the bile of the cat as well as in human T-tube bile after the injection of $S^{35}O_4$. Experiments were also carried out in rats with surgically ligated bile ducts. When radioactive sulfate was injected into these jaundiced animals, labeled conjugated azobilirubin pigments were demonstrable in the urine and serum.

These observations supported the physiologic occurrence of a sulfate containing bilirubin pigment migrating on paper chromatograms in the area of the major conjugated azobilirubin pigments. This indicated that the polar pigment zone was not chemically pure but contained at least two conjugates of azobilirubin, glucuronide and sulfate. The experiments with radioactive sulfate, however, permitted no index as to the quantitative formation or excretion of sulfate versus glucuronide conjugates and therefore the chemical analyses described below and represented in Table I were carried out.

The labeled azopigment eluted from paper chromatograms was treated with a commercial arysulfatase, Mylase-P®, but radioactive sulfate was not liberated by this crude enzyme. However this commercial preparation also did not attack any of the synthetic sulfate derivatives of bilirubin described below. Since mammalian and bacterial sulfatases, in contrast to β-glucuronidase, are quite specific and will attack some sulfate conjugates and not others (13), these results were not unexpected.

Additional evidence for noncarboxyl-linked conjugates of bilirubin

1. Resistance to β-glucuronidase hydrolysis. The demonstration by Schachter (6) that bilirubin glucuronide reacts with hydroxylamine to form a hydroxamate derivative has provided evidence that the glucuronide molecule is linked at the carboxyl group. This is also in keeping with the relative alkali lability of conjugated bilirubin, since carboxyl conjugates are readily split by alkali. However, Billing, Cole and Lathe (3) noted that the incubation of bile in 0.06 N NaOH resulted in the persistence of a definite yet variable amount of alkali stable pigment (5 to 40 per cent). This

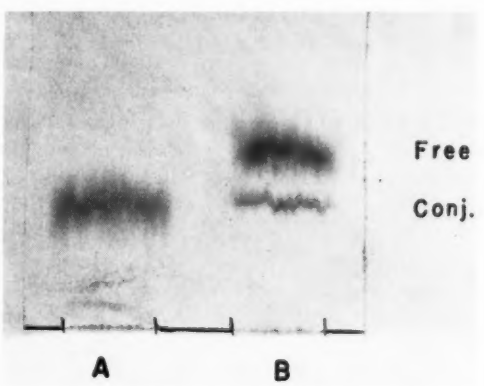

FIG. 3. RESULTS OF β-GLUCURONIDASE TREATMENT OF HUMAN BILE AZOPIGMENTS

Human T-tube bile was diazotized, extracted into butanol and washed as described in text. The azopigments were then exposed to bacterial β-glucuronidase for 24 hours at 37° and then chromatographed in the pyridine system. *A* represents the control which was not incubated. *B* represents the azopigments after β-glucuronidase treatment. It will be seen that some conjugated azopigment remains after enzymatic hydrolysis.

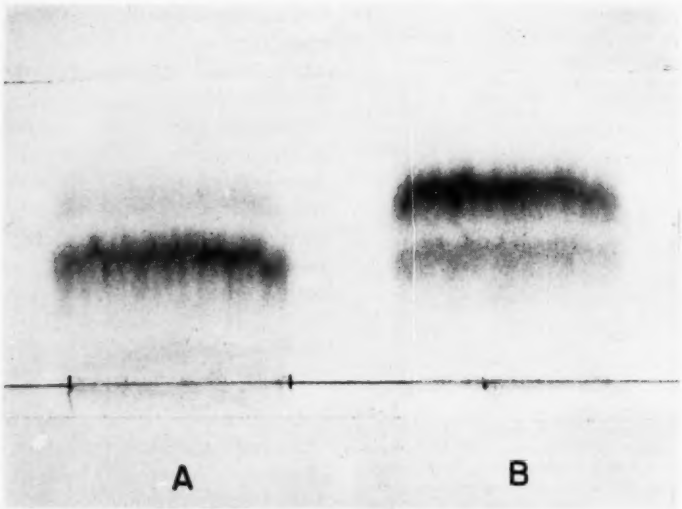

FIG. 4. EFFECT OF ALKALI ON HUMAN BILE AZOPIGMENTS

Bile was diazotized and exposed to a final concentration of 0.1 N NaOH for 10 minutes at 25° C. It was then acidified, washed, extracted into butanol and chromatographed. *A* represents nonalkali treated control. *B* shows residual pigment in conjugated zone after alkali treatment.

suggested that conjugates other than acyl-glucuronides of bilirubin probably existed.

We have repeatedly observed that the incubation of human bile azopigments with bacterial β-glucuronidase results in the persistence of a conjugated azobilirubin pigment on paper chromatography (Figure 3). Since bile itself is inhibitory to β-glucuronidase, the pigments were purified prior to incubation, either by treatment with organic solvents (as described under Methods) or by preliminary paper chromatography and elution. Absence of inhibitors to β-glucuronidase was shown by demonstrating that added phenolphthalein glucuronide was completely hydrolyzed under these conditions. It was realized that the presence of a β-glucuronidase resistant, polar azopigment did not exclude the possibility of an N-glucuronide, a linkage which is not susceptible to hydrolysis by the enzyme. For this reason numerous quantitative chemical analyses were carried out on the polar pigment remaining after incubation with β-glucuronidase. Repeated determinations on different samples failed to detect any glucuronic acid in this residual polar pigment. Table I indicates the results of β-glucuronidase treatment of the azopigments of human T-tube bile obtained from patients with cholelithiasis or common duct stones but without evident liver disease. In 11 samples this β-glucuronidase resistant fraction ranged from 16.7 to 34.6 per cent with a mean of 23.7 ± 6.4 per cent.

2. Alkali stability. We have also carried out studies on the alkali stability of human bile pigments. In view of the fact that an alkaline pH promotes the oxidation of bilirubin and its conjugates to biliverdin derivatives (which do not react with the diazo reagent), the bile pigments were first diazotized and then tested for their alkali stability. Exposure of the azopigments to a final concentration of 0.1 N NaOH for 10 minutes at room temperature resulted in the persistence of a pigment in the polar zone on chromatography (Figure 4). The amounts of this alkali stable pigment have varied from 6.8 to 24.6 per cent with a mean of 15.0 ± 6.2 per cent in 11 different specimens (Table I). As was to be expected, this pigment appeared to possess a free carboxyl group. This was supported by the fact that it did not react with hydroxylamine, but did yield a methyl derivative after treatment with diazomethane. The latter was identified by ascending chromatography in the pyridine system.

Higher concentrations of alkali or longer incubations at room temperature did not appreciably affect the per cent of alkali stable pigment remaining. It will be noted in Table I that our values are lower than those of Billing, Cole and Lathe (3). These authors used a final concentration of 0.06 N NaOH compared to 0.1 N NaOH in the experiments reported here.

3. Hydrolyzable sulfate content of human bile azopigments. The conjugated azopigments isolated by paper chromatography after treatment with either β-glucuronidase or alkali were subjected to acid hydrolysis as described under Methods and the micromoles of inorganic sulfate liberated per micromole of azopigment determined. The results on 11 samples of human T-tube bile (expressed in terms of the total bile azopigments) indicate that 7.0 to 22.6 per cent of the azopigments contained hydrolyzable inorganic sulfate, with a mean of 13.7 ± 5.6 per cent.

Studies with synthetic bilirubin sulfate

Watson has described that bilirubin, when treated with sulfuric acid in the Liebermann-Burchard reaction for cholesterol, is converted to a direct-reacting pigment (14). More recently he has indicated that a sulfate derivative of bilirubin is formed in the reaction (15). We have likewise observed that treatment of bilirubin with sulfuric acid, chlorosulfonic acid or the Liebermann-Burchard reagent results in a product which gives a "direct" Van den Bergh reaction.

When such reactions were carried out in the presence of radioactive sulfate and the products chromatographed, we noted results which suggested that more subtle alterations of the bilirubin molecule probably occurred during the course of these chemical interactions. Figure 5 is a composite of a typical experiment. It is seen in Chromatogram B (Figure 5), that three pigment derivatives are formed, the upper two having the characteristic red-blue appearance of an azopigment while the lower zone was green, and not diazotized. The center pigment zone, it will be noted, migrates with an Rf comparable to the main conjugated pigment zone of bile (Figure 5,A). On the other hand, the autoradiograph of Chromatogram B (Figure 5,C) reveals that after the chemical reaction with sulfuric acid only the

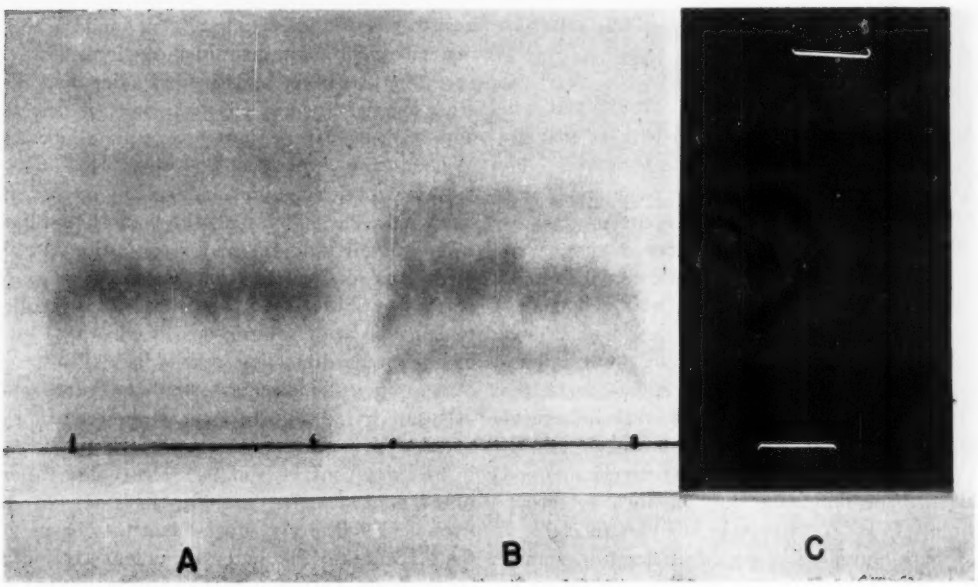

FIG. 5. OBSERVATIONS ON SYNTHETIC "BILIRUBIN SULFATE"

A represents chromatography of normal bile azopigments in which the major band of pigment is conjugated. *B* represents the products obtained after the diazotization of bilirubin which has previously been treated with Liebermann-Burchard reagent containing sulfuric acid and labeled sulfate. *C* is the autoradiograph of *B*.

upper of the three pigment derivatives of bilirubin contains radioactivity (*i.e.*, sulfate). It is to be noted that the other two pigments are polar and water soluble and yet contain no radioactive sulfate. The other radioactive areas in the autoradiograph which do not correspond to any pigment zones are inorganic sulfate (at the origin) and polysulfate. It appears therefore that treatment of bilirubin with sulfuric acid, the Liebermann-Burchard reagent or chlorosulfonic acid results in changes of the pigment molecule which must extend beyond the simple addition of a sulfate radical.

Further evidence for structural alterations of the molecule occurring during the course of these reactions is the fact that when the products are subjected to acid hydrolysis in 6 N HCl, inorganic sulfate is liberated but pigment migrating as free azobilirubin is not detected. This is in distinct contrast to the acid hydrolysis of bile which results in the liberation of azobilirubin, readily identified by paper chromatography.

Finally, the absorption spectrum of the synthetic products (taken together) differs from that of the conjugated azopigments of human bile. The absorption maximum of the former in 0.5 N acetic acid is 550mμ, while that of the latter is 532 mμ.

DISCUSSION

It is apparent from the above studies that although bilirubin glucuronide is the major conjugated bilirubin pigment, other polar and water soluble derivatives of bilirubin occur physiologically. That bilirubin sulfate is one of these conjugates is seen from the experiments with radioactive sulfate injection, analysis of the bile azopigments after exposure to β-glucuronidase and alkali, as well as chemical analysis. On a theoretical basis one would expect a sulfate derivative to be present in an alkali stable ether linkage at one of the hydroxyl groups of bilirubin. This is supported by the studies with hydroxylamine and diazomethane indicating that the carboxyl group is free. It is seen from the analyses in Table I that there must be still another fraction in human bile, amounting to 9 or 10 per cent, which contains neither glucuronide nor sulfate but is polar and alkali labile. Considering other known hepatic conjugation mechanisms (16) it is possible that these conjugates may be methyl or glycine derivatives of bilirubin linked at the carboxyl group. It is certainly evident that the main polar and conjugated azopigment zone as observed in the three paper chromatographic systems is not homogeneous or chemically pure.

The analyses of human T-tube bile for non-glucuronide and sulfate derivatives do not permit a conclusion as to the existence of a monosulfate or a disulfate derivative of bilirubin. It is possible that bilirubin conjugates containing both glucuronic acid and sulfate occur, as in the case of morphine (17). It is furthermore conceivable that in the presence of liver disease, variations in the percentage of excreted glucuronide or sulfate derivatives may take place. *In vitro* observations in our laboratory indicate, for example, that the total glucuronide conjugating capacity of the mammalian liver declines readily with experimental liver damage (18).

Watson has reported that he was not able to detect any inorganic sulfate upon acid hydrolysis of the conjugated azopigment of bile after elution from paper chromatograms (15). However, since the method employed was a gravimetric one, it is very likely that small quantities of hydrolyzable sulfate escaped detection. We found in working with micromole quantities of pigment it was necessary to use the sensitive benzidine method of Dodgson and Spencer (9) to permit the detection and analysis of the inorganic sulfate liberated. Watson also expressed the view (15) that a naturally occurring bilirubin sulfate seemed unlikely since the Rf of synthetic "bilirubin sulfate" was different from the major conjugated azopigment zone on paper chromatography. For the reasons outlined above, we believe that treatment of bilirubin with sulfuric acid, as in the Liebermann-Burchard reaction, results in subtle changes of the pigment molecule beyond the introduction of a sulfate radical. In fact, as observed in Figure 5, only one of the polar derivatives formed in this way contains sulfate. These observations, plus the fact that free azobilirubin is not liberated upon acid hydrolysis, suggest that the disparity in the Rf values of the natural azopigments and the synthetic product cannot be used to exclude the physiologic occurrence of bilirubin sulfate.

Additional evidence for the existence of a bilirubin sulfate conjugate is the fact that we have

recently obtained evidence for the enzymatic synthesis of such a conjugate (19). This reaction has been observed in an ammonium sulfate fraction of rat liver. As was to be expected from the data of others on enzymatic sulfate conjugation (20), this system requires adenosine triphosphate. The "active sulfate" (adenosine-3′ phosphate-5′ phosphosulfate) described by Robbins and Lipmann (21) is probably an intermediate in this synthesis.

SUMMARY

1. In addition to bilirubin glucuronide, nonglucuronide conjugates of bilirubin that are polar and water soluble occur physiologically. One of these conjugates, bilirubin sulfate, can readily be identified in the bile of rats, cats or man after the injection of radioactive sulfate. In contrast to bilirubin glucuronide, where the glucuronide is attached at the carboxyl groups, chemical studies suggest that the sulfate is linked at the hydroxyl groups of the pigment.

2. Additional evidence for the occurrence of nonglucuronide conjugates of bilirubin is provided by the presence of a conjugated azobilirubin fraction which resists β-glucuronidase hydrolysis and contains no glucuronic acid. A part of this fraction is stable to alkali.

3. Analyses of the bilirubin azopigments in 11 human T-tube bile samples revealed a mean β-glucuronidase resistant fraction of 23.7 ± 6.4 per cent, a mean bilirubin sulfate content of 13.7 ± 5.6 per cent and an alkali stable residue with a mean of 15.0 ± 6.2 per cent. These data suggest that there is yet another conjugated azobilirubin fraction amounting to 9 or 10 per cent, which is alkali labile and contains neither glucuronic acid nor sulfate.

4. Organic reactions can be used to form sulfate derivatives of bilirubin but they have the limitation of also producing other structural alterations in the bilirubin molecule.

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THE RENAL EXCRETION OF HEMOGLOBIN: REGULATORY MECHANISMS AND THE DIFFERENTIAL EXCRETION OF FREE AND PROTEIN-BOUND HEMOGLOBIN * †

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Previous studies of the transport and excretion of extracorpuscular hemoglobin by the kidney have been based on the assumption that hemoglobin circulates in plasma in the free, unbound state, and within the limitations imposed by the glomerular membrane, freely diffuses into glomerular filtrate. The demonstration (2, 3) that plasma proteins possess the property of binding hemoglobin and that hemoglobin may circulate in both the free and unbound states (4–6) has invalidated the conceptual basis of these studies. Consequently many of the previous concepts of renal hemoglobin transport are no longer tenable and must be revised.

Observations of hemoglobin binding *in vivo* and *in vitro* have established that the capacity of plasma proteins to bind hemoglobin is limited (3–6). Since this capacity is near the plasma level at which hemoglobinuria characteristically occurs, it has been suggested (4, 5) that the limitation imposed on hemoglobin excretion at low plasma levels is related to protein-binding and that only when the binding capacity is exceeded and hemoglobin circulates in the free, unbound state does hemoglobinuria occur. Implicit in this suggestion is the assumption that free and protein-bound hemoglobin are transported differently by the kidney. This assumption has not, however, been subjected to experimental study and the respective roles these transport phenomena play in regulating hemoglobin excretion is unknown. The present investigation was therefore undertaken for the purpose of studying these aspects of renal hemoglobin transport and of re-evaluating the regulatory mechanisms of hemoglobin excretion in man.

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† Presented at the annual meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 5, 1958 (1).

METHODS

Renal hemoglobin excretion and transport were measured under conditions of a progressivly increasing plasma hemoglobin concentration in 10 adult human subjects (aged 18 to 48 years) who were free of apparent renal, cardiovascular, hematologic or other disease. All subjects were fasting at the time of the study and each had ingested 500 ml. of water approximately one hour prior to the test. A catheter was placed in the urinary bladder and an indwelling needle was inserted in the brachial artery. Arterial blood was withdrawn and hemolyzed according to methods previously described (6), and priming and sustaining infusions of hemoglobin were prepared. The priming infusions contained approximately 2.0 Gm. of hemoglobin in 50 ml. of solution. The sustaining infusion contained 2.0 Gm. of hemoglobin and 50 ml. of inulin in 1,000 ml. of 5 per cent dextrose in water. Immediately after preparation of these solutions the first of from five to seven priming injections of hemoglobin was given intravenously, along with 25 ml. of inulin, and the sustaining infusion was started. The sustaining infusion was administered intravenously at a rate of 4.5 ml. per minute (9.0 mg. of hemoglobin per minute) throughout the study. Additional priming injections of hemoglobin were given at 45 to 60 minute intervals throughout the study. These injections were given as rapidly as possible, usually over a period of from five to 10 minutes, without causing undue discomfort over the site of the vein used for injection. The total hemoglobin administered was approximately 14 to 18 Gm., given over a period of four hours.

After each priming injection of hemoglobin a period of from 15 to 30 minutes was allowed for adjustments of plasma and urinary levels, and the urine was discarded. Urine was then collected for analysis during two consecutive 15 to 20 minute periods. At approximately the midpoint of each period a sample of arterial blood was withdrawn. Standard renal clearance techniques were used for collection of the urine. The arterial blood samples were collected with care to avoid hemolysis and were heparinized and centrifuged immediately. Analysis of plasma and urine was usually made on the day of the study. When analysis was delayed plasma and urine were frozen and stored at $-10°$ C.

Plasma and urine were analyzed for inulin by the method of Harrison (7) and the clearance of inulin during each collection period was calculated. The total hemoglobin content of plasma and urine was determined by the method of Evelyn and Malloy (8). The concentrations of free hemoglobin, protein-bound hemoglobin and methemalbumin in plasma and urine were determined by a paper electrophoretic method previously described (6). This method consisted of the separation of these moieties on paper after overnight electrophoresis utilizing a phosphate buffer, pH 7.0, ionic strength 0.05. Following electrophoresis the individual fractions were identified by staining with 1 or 2 per cent benzidine in 20 per cent acetic acid and 3 per cent hydrogen peroxide. The appearance of oxidized benzidine in the areas of the paper containing hemoglobin (or methemalbumin) permitted visualization of the separated fractions which were identified by their characteristic positional relationships.

Quantification of the individual fractions was made by photometric analysis (Spinco Analytrol®) of the stained filter paper strips, where measurements of light absorption were used to calculate the relative concentrations of each fraction. The details of these techniques have been described previously (6).

The data obtained from these analyses were used to evaluate the relationships between plasma and urinary hemoglobin and to measure two parameters of renal hemoglobin transport: glomerular clearance and tubular reabsorption. The calculations employed in these measurements are presented under Results.

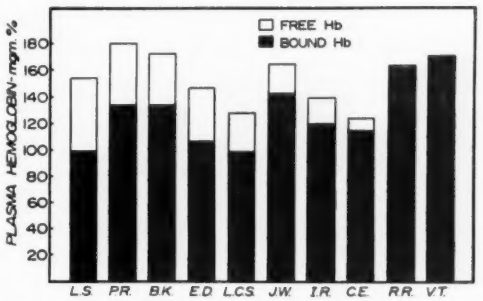

FIG. 1. THE RELATIONSHIP BETWEEN THE CONCENTRATION OF HEMOGLOBIN IN PLASMA AND THE APPEARANCE OF HEMOGLOBIN IN THE URINE

Each bar represents an individual experiment with the subject's initials at the bottom of the figure. The top of the bar represents the estimated total concentration of hemoglobin in plasma which was attained prior to the appearance of hemoglobin in the urine. The components of total hemoglobin in plasma are represented by the clear and solid portions of the bars, where the clear portion represents the concentration of free hemoglobin and the solid portion the concentration of protein-bound hemoglobin (maximal binding capacity). In each instance hemoglobinuria did not occur until the maximal binding capacity was exceeded and free, unbound hemoglobin appeared in plasma. In Subject V.T., hemoglobinuria would theoretically occur at any concentration of free hemoglobin in plasma.

RESULTS

Plasma hemoglobin

Following each priming infusion the concentration of hemoglobin in plasma increased. Between priming injections the concentration remained relatively constant. The hemoglobin administered during the early phases of the study was quantitatively bound to plasma protein and circulated entirely in this state. As the plasma level increased, the concentration of protein-bound hemoglobin rose progressively until a level was attained at which additional binding did not occur and hemoglobin appeared in plasma in the free, unbound state. This level, which may be considered as the maximal binding capacity of plasma protein for hemoglobin, varied in individual studies from 98 to 171 mg. per cent and averaged 128 ± 25 mg. per cent. As the plasma level of hemoglobin increased above the maximal binding capacity the concentration of free hemoglobin progressively rose. In all but two subjects (V. T. and E. D.) small quantities of methemalbumin (5 to 11 mg. per cent) appeared in plasma coincident with the appearance of free hemoglobin. The level of methemalbumin remained relatively constant throughout the study. At the conclusion of the test the total hemoglobin concentration in plasma varied in individual studies from 205 to 355 mg. per cent and the concentration of free hemoglobin from 101 to 263 mg. per cent.

Urinary hemoglobin

As the plasma hemoglobin concentration increased the urine remained free of hemoglobin until the maximal binding capacity of plasma proteins was exceeded and hemoglobin circulated in the free, unbound state (Figure 1). With the appearance of free hemoglobin in plasma, hemoglobin appeared in the urine. Electrophoretic studies of urinary hemoglobin at all plasma levels and excretory rates obtained revealed that in each subject the electrophoretic characteristics of urinary hemoglobin were in all respects similar to those of free hemoglobin in plasma (Figure 2). At pH 7.0 both urinary hemoglobin and plasma free hemoglobin remained at the site of application

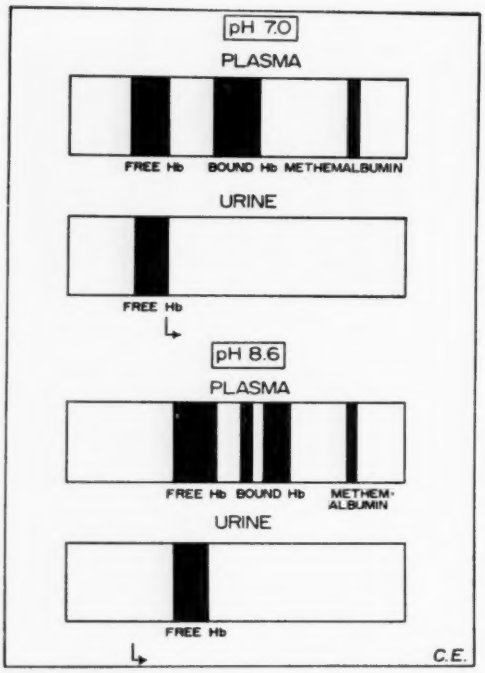

FIG. 2. THE PAPER ELECTROPHORETIC CHARACTERISTICS OF PLASMA AND URINARY HEMOGLOBIN

The positions of the various heme-fractions are shown schematically at pH 7.0 in the upper part of the figure and at pH 8.6 in the lower part of the figure. The arrows refer to the application site and direction of movement towards the anode. At both pH 7.0 and 8.6 the electrophoretic characteristics of urinary hemoglobin were the same as those of plasma free, unbound hemoglobin. The position that free hemoglobin assumes to the cathode side of the application site at pH 7.0 is due to endosmotic flow. Protein-bound hemoglobin and methemalbumin were absent from the urine. In plasma, protein-bound hemoglobin appears as two separate fractions at pH 8.6 and as one fraction at pH 7.0.

of the sample to the paper; at pH 8.6 urinary hemoglobin migrated at the same speed as plasma free hemoglobin. Thus, the circulation of free hemoglobin in plasma was associated with the appearance in the urine of hemoglobin which was, in terms of its electrophoretic characteristics, similarly free and unbound. Only free hemoglobin was excreted; in no instance was PBH or methemalbumin excreted in detectable amounts.

From a study of the relationship between changing excretory rates and plasma levels of free hemoglobin (Figure 3) estimates were made of the theoretical plasma level of free hemoglobin at which hemoglobinuria occurred. These estimates were made by an extrapolation of the curves (UV/P) shown in Figure 3, where the extrapolated intercept of the horizontal (P) axis was taken as the plasma concentration of free hemoglobin above which hemoglobin appeared in the urine. In all experiments the lowest determined value of UV used in this extrapolation was less than 1.0 mg. per minute. Thus the horizontal axis was intercepted by a short extension from determined values. Although this lessened the possibility of erroneous estimation of P, the values obtained are considered as theoretical estimates only since the relationship between UV and P at low values of each was undetermined. Within

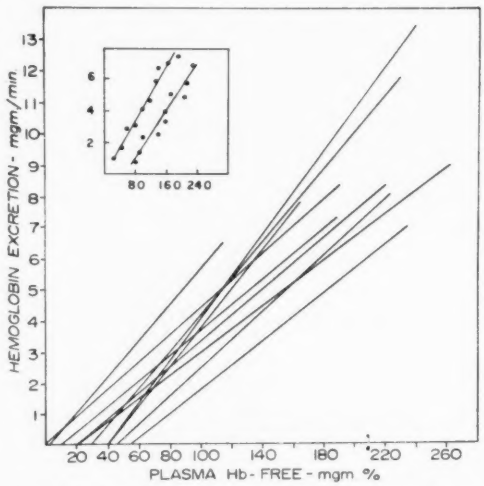

FIG. 3. THE RELATIONSHIP BETWEEN THE EXCRETORY RATE AND PLASMA CONCENTRATION OF FREE HEMOGLOBIN

Each curve represents an individual experiment. The lower portion of the curves which intercept the horizonal axis was obtained by extrapolation from determined values. In each instance such extrapolation was made from a determined excretory rate of less than 1.0 mg. per minute. The intercept of the horizontal axis represents the theoretical plasma level of free hemoglobin above which hemoglobinuria occurred. The slope of the curves (Δ UV/Δ P) represents the glomerular clearance of free hemoglobin. The determined values of UV and P from which curves were constructed in two of the experiments are shown in the insert. These two experiments represent those with the lowest and highest extrapolated P value. In all experiments the curves were drawn by visual approximation from determined values of UV and P.

these limitations and that imposed by the accuracy of estimating the relationship of UV:P, the values obtained for P, shown in Figures 1 and 3, varied in individual studies from 0 to 54 mg. per cent and averaged 27 ± 19 mg. per cent. When these levels of free hemoglobin were added to those of the respective maximal concentrations of protein-bound hemoglobin, the estimated maximal plasma level of total hemoglobin which was attained before hemoglobin appeared in the urine varied from 124 to 180 mg. per cent (Figure 1). These levels are in agreement with previous estimates of the relationship between plasma hemoglobin (total) concentration and the development of hemoglobinuria (9).

Glomerular clearance of free hemoglobin

The urinary excretion of free hemoglobin increased progressively and proportionately as the plasma level of free hemoglobin increased (Figure 3). Using this relationship, the rate at which free hemoglobin was cleared from plasma at the glomerulus was calculated from a plot of UV against P, where the slope of the curves obtained represented the clearance rate ($C^G_{\text{Free Hb}}$) in ml. per minute:

$$\text{Glomerular clearance (ml. per min.)} = \frac{\Delta U_{\text{Free Hb}} V}{\Delta P_{\text{Free Hb}}}. \quad 1)$$

When corrected for body surface area the glomerular clearance of free hemoglobin varied in the group of 10 subjects from 3.0 to 7.0 and averaged 5.0 ± 1.0 ml. per minute per 1.73 square meters body surface area (Table I).

The curves used to calculate these values were those constructed from determined and not from extrapolated values of UV and P. The plasma level of free hemoglobin used in these calculations was obtained by extrapolation for intrarenal delay to a point 2.5 minutes before the midpoint of each collection period. In practice such an extrapolation was seldom necessary since the plasma level changed only slightly during the 30 to 60 minute period between priming injections.

The clearance of inulin in these studies varied from 77 to 122 ml. per minute per 1.73 square meters. These values are lower than those reported in normal, basal subjects (10). This may be attributed to renal vasoconstriction with reduction in renal blood flow and glomerular filtration rate which accompanies the administration of hemoglobin intravenously (11). Progressive changes in glomerular filtration rate with each priming injection, as have been observed by others (11), did not occur and the inulin clearance remained constant throughout each study.

TABLE I

The glomerular clearance of free hemoglobin and the renal clearance of inulin *

Subject	C^G_{Hb}	C_{In}	C^G_{Hb}/C_{In}
	ml./min.	*ml./min.*	
B. K.	7	101	0.066
R. R.	6	96	0.063
I. C. S.	6	91	0.063
E. D.	5	91	0.056
V. T.	5	122	0.039
C. E.	5	87	0.053
I. R.	4	84	0.052
P. R.	4	104	0.038
L. S.	4	97	0.040
J. W.	3	77	0.043
Mean $\pm$ S. D.	5 ± 1		0.051 ± 0.01

* All values are corrected to 1.73 sq. meters body surface area. Abbreviations are as follows: C^G_{Hb} the glomerular clearance of free hemoglobin; C_{In}, the renal clearance of inulin.

The ratio $C^G_{\text{Free Hb}}/C_{In}$, tabulated in Table I, averaged 0.05 ± 0.01. This ratio expresses the glomerular permeability to hemoglobin relative to inulin.

Tubular reabsorption of free hemoglobin

The rate of tubular reabsorption of free hemoglobin was calculated as the difference between the filtered load and excretory rate at any given plasma level of free hemoglobin:

$$\text{Filtered load (mg. per minute)} = C^G_{\text{Free Hb}} \times P_{\text{Free Hb}}. \quad 2)$$

$$\text{Reabsorptive rate (mg. per minute)} = C^G_{\text{Free Hb}} \times P_{\text{Free Hb}} - U_{\text{Free Hb}} V. \quad 3)$$

At loads sufficient to induce hemoglobinuria, the relationship between the excretory rate and plasma level was linear (Figure 3) and the calculated reabsorptive rate did not, therefore, change with increasing loads. Hence, the calculated reabsorptive rate at these loads may be considered as the estimated maximal reabsorptive capacity. This capacity, obtained at load/T ratios in excess of 3.0 in all subjects, varied in individual subjects from 0.0 to 2.6, and averaged 1.3 ± 0.9 mg. per minute

TABLE II

The estimated maximal rate of tubular reabsorption of free hemoglobin *

Subject	T_{Hb}	T_{Hb}/100 ml. filtrate	C^{G}_{Hb}/T_{Hb}
	mg./min.	*mg./100 ml.*	*ml./mg.*
B. K.	2.6	2.6	2.6
E. D.	2.1	2.3	2.4
L. S.	2.1	2.2	1.9
P. R.	1.9	1.8	2.1
L. C. S.	1.6	1.8	3.6
I. R.	0.8	1.0	5.5
J. W.	0.8	1.0	4.1
C. E.	0.4	0.5	11.5
R. R.	0.3	0.3	20.0
V. T.	0.0	0.0	
Mean ± S. D.	1.3 ± 0.9	1.4 ± 1.0	6.0 ± 6.0

* All values are corrected to 1.73 sq. meters body surface area. Abbreviations are as follows: T_{Hb}, calculated maximal tubular reabsorptive rate of free hemoglobin (mg. per minute); T_{Hb}/100 ml. filtrate, mg. of free hemoglobin reabsorbed per 100 ml. of glomerular filtrate; C^{G}_{Hb}, the glomerular clearance of free hemoglobin (ml. per minute).

per 1.73 square meters body surface area (Table II). In five subjects (I.R., J.W., C.E., R.R. and V.T.) the estimated reabsorptive capacity was less than 1.0 mg. per minute and in five subjects (B.K., E.D., L.S., P.R. and L.C.S.) greater than 1.0 mg. per minute. The intercept of the curves obtained from a plot of UV against P was at or near zero in those subjects with a low estimated reabsorptive capacity and was displaced from zero in those subjects with a reabsorptive capacity in excess of 1.0 mg. per minute (Figure 3). There was no correlation between estimated reabsorptive capacity and glomerular clearance (Table II).

When reabsorption was expressed in terms of glomerular filtration rate, an average of 1.4 ± 1.0 mg. of hemoglobin was reabsorbed per 100 ml. of glomerular filtrate (Table II).

DISCUSSION

This study has demonstrated, as predicted (4, 5), that hemoglobin is not excreted in the urine until the plasma hemoglobin concentration reaches a level which exceeds the capacity of plasma proteins to bind hemoglobin. When this capacity, which averages 128 mg. per cent, is exceeded, free, unbound hemoglobin appears in plasma and is excreted in the urine. Under these circumstances, and as the plasma level changes, the urine contains free hemoglobin, but does not contain protein-bound hemoglobin, which is not excreted.

The rate at which free hemoglobin is excreted is dependent upon three variables: *1*) the plasma level of free hemoglobin, *2*) the rate of clearance from plasma by the glomerulus, and *3*) the rate of tubular reabsorption. Under conditions of a progressively increasing plasma concentration of free hemoglobin the excretory rate increased in the present study in proportion to the change in plasma level. At any given plasma level the rate of excretion appeared to depend in large measure upon the rate at which free hemoglobin was filtered by the glomerulus and only to a negligible, if any, extent on tubular reabsorptive activity. Estimates of glomerular clearance made from the relationship between the changing excretory rate and plasma concentration ($\Delta UV/\Delta P$) revealed that on the average 5 ml. of plasma was cleared of free hemoglobin per minute by the glomerulus. Since the clearance of inulin averaged approximately 100 ml. per minute, the glomerulus was 5 per cent as permeable to free hemoglobin as to water (inulin). Or, expressed in another way, the concentration of free hemoglobin in glomerular filtrate averaged 5 per cent of that in plasma. The permeability of the glomerulus to hemoglobin is therefore much less than previous estimates which were made without consideration of the physicochemical state of plasma hemoglobin (9).

Although the glomerular clearance was small, considerable amounts of hemoglobin were filtered. Calculated filtered loads reached 8 to 10 mg. per minute in most studies. It appeared that most, if not all, of this filtered free hemoglobin was excreted in the urine. The highest estimated maximal tubular reabsorptive rate observed was 2.6 mg. per minute, and in one-half of the subjects the rate was less than 1.0 mg. per minute. Thus the composition of glomerular filtrate with respect to free hemoglobin appeared to change but little during the passage of urine down the renal tubules. Indeed, the values obtained for maximal reabsorptive capacity are so small that it is uncertain whether the composition changed at all. The physiologic validity of the calculations of reabsorptive rate is dependent upon an accurate determination of two variables: load and excretory rate. Since these variables were large in relation to the differences between them (reabsorptive rate), inaccuracies in the determination of either would affect to a con-

siderable extent estimates of tubular reabsorptive activity, the physiologic significance of which would depend upon the direction and magnitude of the errors involved. Without knowing the extent and direction of such errors, the values obtained for reabsorptive capacity appear to be of insufficient magnitude in relation to possible errors in their estimation to justify definite conclusions regarding the quantitative aspects of tubular reabsorption. The results are consistent with a low rate of tubular reabsorption of free hemoglobin but do not distinguish between small or absent reabsorptive activity.

If a precise quantitative estimate of tubular reabsorption cannot be made, these observations indicate that tubular reabsorption of free hemoglobin is at least small and does not play a determinant role in regulating hemoglobin excretion. It would therefore be anticipated that hemoglobin would appear in the urine at low plasma levels of free hemoglobin. This was, in fact, observed in the present study where estimates of the plasma level above which hemoglobinuria occurred averaged 27 mg. per cent. Since, however, calculations of this value also depended upon an accurate estimation of the slope $\Delta UP : \Delta P$, and upon an extrapolation as well, this figure must be interpreted cautiously. Nonetheless, in several subjects this estimated plasma level exceeded 20 mg. per cent and in one subject approached 60 mg. per cent (Figure 3), values which would be difficult, because of their magnitude, to attribute to errors in estimating the slope alone. These figures are therefore more suggestive of tubular reabsorptive activity than are the calculated reabsorptive rates, on the assumption that it was necessary to elevate the plasma concentration of free hemoglobin to these levels before tubular reabsorptive activity was saturated and hemoglobinuria occurred.

These considerations are not conclusive, however, and the evidence for tubular reabsorption in man remains doubtful. Although experiments in animals suggest that hemoglobin finds its way into renal tubular cells (12–14) the evidence in man is less convincing and appears to consist principally of the demonstration of hemosiderin in renal tubular cells (15), in the urine and in urinary casts (16) in patients with hemolytic disorders. There is little histologic evidence of tubular reabsorption in man (17) and the present study does not provide unequivocal physiologic evidence. More definitive studies are therefore necessary before it can be concluded that tubular reabsorption of free hemoglobin occurs in man.

The failure to detect protein-bound hemoglobin or methemalbumin in the urine in the present study when these constituents were present in plasma may be attributed to lack of filtration or to complete tubular reabsorption of these hemeproteins. In view of what is known concerning glomerular permeability and tubular reabsorption of large molecular substances, these heme-protein complexes would be expected to traverse the glomerular membrane with difficulty and would be unlikely to appear in glomerular filtrate in significant concentration under normal circumstances (18, 19). The molecular weight and size of methemalbumin is certainly equal to that of albumin (20), to which the normal glomerulus is believed to be in large measure impermeable (18, 19). Although the physical characteristics of protein-bound hemoglobin have not been definitely established, the molecular weight of this hemoglobin-α_2 globulin (?) complex appears to be in excess of 300,000 (4), suggesting that it also is unlikely to traverse the glomerular membrane readily because of its size. These considerations therefore suggest that the limitation imposed upon the excretion of these substances is attributable, at least in large part, to the permeability characteristics of the glomerular membrane rather than to avid tubular reabsorption activity.

The probem raised by Smith (10) as to why the glomerulus is as permeable as it appears to be to (free) hemoglobin, which in molecular weight, at least, is similar to albumin (20), is not resolved. The lower estimates of glomerular permeability made in this study are more consistent than previous estimates (9) with the molecular weight and physical characteristics of free hemoglobin (20). However, the question is unanswered as to whether the glomerulus is permeable to the free hemoglobin molecule as such or whether hemoglobin enters glomerular filtrate only after dissociating into smaller parts.

SUMMARY

A study of urinary hemoglobin excretion and renal hemoglobin transport was made in 10 normal

human subjects. Under conditions of a progressively increasing plasma hemoglobin concentration, hemoglobinuria did not occur until the plasma level exceeded the capacity of plasma proteins to bind hemoglobin. When this capacity, which averaged 128 mg. per cent, was exceeded, free, unbound hemoglobin appeared in plasma and was excreted in the urine.

A linear relationship was demonstrated between the excretory rate and plasma level of free hemoglobin as the plasma concentration increased. From this relationship calculations were made of the glomerular clearance and tubular reabsorption of free hemoglobin. The glomerular clearance averaged 5 ml. per minute per 1.73 square meters of body surface area and averaged 5 per cent of the inulin clearance. The estimated maximal reabsorptive rate averaged 1.0 ml. per minute per 1.73 square meters of body surface area. This value was too small to be considered as conclusive evidence of tubular reabsorptive activity.

Neither protein-bound hemoglobin nor methemalbumin was excreted in the urine when these constituents were present in plasma. This was attributed to a failure of these large heme-proteins to traverse the glomerular membrane.

ACKNOWLEDGMENTS

The author is indebted to Mrs. Mary Elizabeth Sweeney and Miss Carolyn Patton for technical assistance.

ADDENDUM

Since this work was completed Vanderveiken, Gueritte, de Myttenaere and Lambert have reported (Effets de la liason hemoglobine-haptoglobine sur l'excretion de l'hemoglobine. J. d'Urol. 1958, **64**, 137) on a failure to find physiologic evidence of tubular reabsorption of free hemoglobin in the dog.

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THE EFFECT OF BODY POSITION CHANGE ON LUNG COMPLIANCE IN NORMAL SUBJECTS AND IN PATIENTS WITH CONGESTIVE HEART FAILURE *

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Since the work of Christie and Meakins (1), the lungs of patients with heart failure have been acknowledged to resist inflation to a greater extent than normal lungs. More recent studies of pulmonary mechanics in patients with congestive heart failure (2–5) have emphasized the lowered lung compliance as well as moderate increases in pulmonary resistance. That acute changes in lung compliance can occur in man has been shown by Saxton, Rabinowitz, Dexter and Haynes (6) who demonstrated acute decreases in lung compliance on exercise in patients with heart disease associated with increases in pulmonary artery wedge pressure, and by Bondurant, Hickam and Isley (7) who demonstrated acutely decreased compliance in normal subjects when transient pulmonary congestion was induced by sudden inflation of a pressurized suit. Because of the importance of body position in cardiac dyspnea, a feature of which is orthopnea, the present study was undertaken to determine what, if any, changes in the static volume-pressure characteristics of the lungs occurred in normal subjects and in orthopneic patients with heart failure on changing from the sitting to the horizontal (prone) body position.

METHODS

The data herein presented were derived from simultaneous oscillographic recordings [1] of transpulmonary pressure, air flow and the tidal volume. Esophageal pressure was recorded by means of a balloon catheter (balloon length, 15 cm.) similar to that described by Mead, McIlroy, Selverstone and Kriete (8). It was inflated with 1 ml. of air when in use. This catheter was attached to one side of a differential pressure transducer [2] of low volume displacement with small and equal air volumes on the two sides of the diaphragm. The other side of the transducer was connected to the mouthpiece so that the pressure recorded represents the difference between pressures in the mouth and the esophagus or the transpulmonary pressure. The 95 per cent response time of this system was approximately 0.005 second and its natural frequency, 100 cycles per second. Airflow was recorded by measuring the differential pressure across a 400-mesh monel metal screen two inches in diameter by means of a differential pressure transducer [3] also provided with small and equal volumes on either side of the diaphragm. The 95 per cent response time of the flow recording system was 0.015 second and its natural frequency, 40 cycles per second. Tidal volume was recorded in early experiments on a 7 L. spirometer.[4] To the spirometer counterweight was attached an electrical contact to a resistance wire which formed two arms of a resistance bridge activated by a mercury cell; the output of this bridge was fed into a DC amplifier. The output of this volume transducer was linearly related to volume. In later experiments a low resistance, low inertia Krogh spirometer [5] was used, at the fulcrum of which was attached an angular displacement transducer,[6] the output of which was also linearly related to volume. Airflow was interrupted by a specially constructed rotary interrupter valve.[7] The rate of interruption was five interruptions per second with equal "on" and "off" times of 0.1 second. The transition time between flow and interruption was one-sixth of the half-cycle time. Figure 1 is a recording of transpulmonary pressure, tidal volume and airflow illustrating the general form of the tracings from which data were derived and showing the "square wave" characteristics at interruption in the upper pressure curve. It is seen that airflow falls substantially to zero during

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† Established Investigator of the American Heart Association and Buswell Fellow in Medicine.

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[1] Recorder manufactured by Electronics for Medicine Incorporated, White Plains, N. Y.

[2] The Sanborn Company 267B pressure transducer.

[3] Built by Mr. Frederick E. Wiedeman, Department of Physiology, Harvard University School of Public Health.

[4] The Collins Company respirometer.

[5] Fred J. Christensen, Watertown, Mass.

[6] Minneapolis Honeywell microsyn position indicator.

[7] Adapted from a design originated by Dr. John A. Clements.

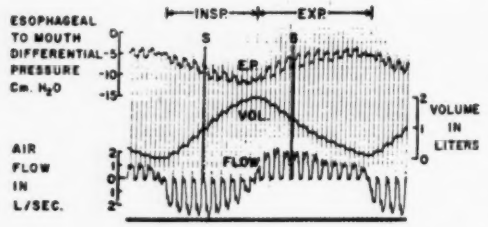

FIG. 1. OSCILLOGRAPHIC TRACING OF TRANSPULMONARY PRESSURE, TIDAL VOLUME AND AIRFLOW IN NORMAL SUBJECT 4

Time lines are 0.1 second; vertical dashed lines labeled S indicate points at which airflow is interrupted or static points. Static volume-pressure curves are obtained by plotting static pressure points against static volume points (the level steps in the middle tracing).

the static (interrupted) phase. Rapid interruption as described was used in this study because it was better tolerated by breathless subjects and enabled an entire volume-pressure curve of many points to be taken during one breath, shortening the time and effort necessary to obtain volume-pressure data.

Though true static pressure equilibration was probably not attained throughout the lung at this rapid rate of interruption, conditions closely approaching static equilibrium were obtained, certainly in normal subjects and probably in patients with heart failure, as evidenced by the observations shown in Figures 2 and 3 that the volume-pressure curves obtained by interruption at five interruptions per second closely resembled those obtained during slower interruptions. The rate at which static equilibration is approached is affected by the magnitude of airway resistance. Unpublished observations made by Clements and associates (9) and the author indicate that equilibration should be virtually complete at this rate of interruption up to resistance values of 10 cm. H_2O per L. per second. In none of the patients was this resistance value exceeded (Table I).

On each of eight healthy young normal subjects and in seven of the eight patients with congestive failure, volume-pressure data were collected in the sitting position and immediately after changing to the prone position. Lung compliance, expressed in L. per cm. H_2O, was taken as the slope of the static volume-pressure curve between the resting end-expiratory volume and 1 L. above this volume. Each compliance value is an average from 10 breaths. Because of observations that lung compliance varies depending on the size of preceeding breaths (10), volume-pressure measurements were made only on maximal breaths encompassing the entire inspiratory capacity (from resting end-expiratory volume to full lung capacity). As it is known that compliance changes with frequency in the presence of unequal time constants of ventilated units (11), the frequency of interruption was kept the same (five per second in all studies) and an effort was made to record all volume-pressure data at the same breathing frequency in each subject. Tidal volume and frequency data are included with compliance values

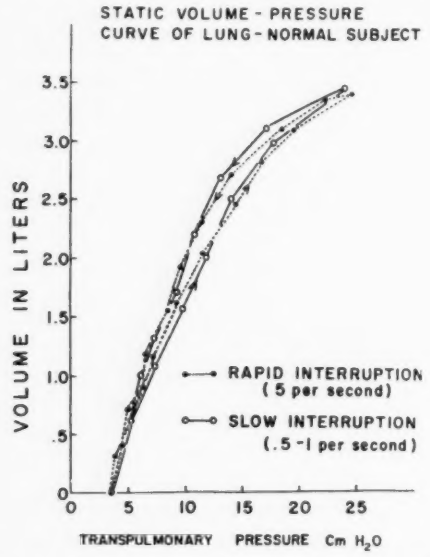

FIG. 2. STATIC VOLUME-PRESSURE CURVES ON A NORMAL SUBJECT DONE AT TWO RATES OF INTERRUPTION AS INDICATED

Dashed lines with solid points indicate interruption at five per second; solid lines with open points indicate flow interruption voluntarily performed by the subject one-half to one time per second. The two curves are hardly distinguishable.

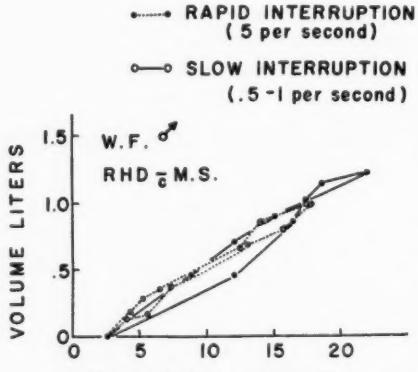

FIG. 3. STATIC VOLUME-PRESSURE CURVES ON PATIENT 2 DONE AT TWO RATES OF INTERRUPTION

Open points with dashed lines, rapid interruption at five per second; solid points with solid lines, slow interruption voluntarily performed by the patient. The two curves yield essentially the same compliance.

TABLE I

Changes in lung compliance with body position change

Subject	Sitting				Prone				
	Compliance ± S. E.	Pulmonary resistance	Tidal volume	Frequency	Compliance ± S. E.	Tidal volume	Frequency	Compliance change	p
	L./cm. H_2O	*cm. H_2O/L./sec.*	*ml.*	*breaths/min.*	*L./cm. H_2O*	*ml.*	*breaths/min.*	%	
Normals									
1. J. S.	.257 ± .005	1.9	3,080	8.4	.267 ± .012	2,920	8.7	+ 3.9	>.05
2. D. Rn.	.115 ± .007	1.8	3,030	6.4	.115 ± .005	2,590	14.5	0	>.05
3. A. B.	.431 ± .045	1.1	3,770	9.1	.458 ± .034	4,660	7.9	+ 5.9	>.05
4. G. G.	.184 ± .008	1.3	2,270	8.1	.171 ± .005	1,480	7.9	− 7	>.05
5. L. W.	.239 ± .008	1.2	2,430	8.3	.229 ± .012	2,020	5.6	− 4.2	>.05
6. M. M.	.178 ± .015	2.5	2,120	12.2	.181 ± .007	2,530	10.1	+ 1.6	>.05
7. T. S.	.096 ± .003	2.3	3,520	8.5	.153 ± .007	2,990	7.9	+38.0	<.05
8. D. Rk.	.308 ± .009	2.3	3,020	5.6	.327 ± .029	2,870	6.5	+ 6.2	>.05
Congestive failure									
1. T. D.	.074 ± .0003	4.7	1,360	11	.054 ± .007	1,040	19	−27	<.01
2. W. F.	.055 ± .002	5.0	1,280	14	.051 ± .002	1,010	17	− 7	>.05
3. F. G.	.100 ± .006	3.6	930	23	.072 ± .002	830	17	−28	<.05
4. C. M. St. 1	.099 ± .003	3.9	1,840	12	.085 ± .004	1,670	13	−14	<.01
St. 2	.125 ± .004	3.1	2,000	18	.103 ± .006	1,780	15	−17.6	<.01
5. J. N.	.082 ± .007	5.6	940	25	.056 ± .004	920	28	−32	<.01
6. W. W.	.082 ± .005	1.7	1,210	13	.063 ± .001	1,120	24	−24	<.01
7. T. J.	.071 ± .001	5.7	460	21	.056 ± .002	490	23	−21	<.01
Congestive failure—Recovery									
5. J. N.	.082 ± .0016	4.2	1,140	26.4	.092 ± .0031	1,090	21.4	+11	>.01, <.05
6. W. W.	.101 ± .0023	2.4	1,680	15.8	.095 ± .0022	1,310	18.9	− 6	>.05

in Table 1. In all patients the catheter was taped tightly to the nose and face to keep the esophageal balloon catheter in the same location throughout the study. Pulmonary resistance was calculated from uninterrupted breaths by the method of von Neergaard and Wirz (12). Values shown are averages from five breaths and were calculated at points where the air flow rate was 1 L. per second.

Observations made by Knowles, Hong and Rahn (13) suggesting deviation of esophageal pressure from pleural pressure below resting end-expiratory volume prompted the authors to make all volume-pressure measurements above the resting end-expiratory volume. This, however, shifted with body position both in normal subjects and in patients. These shifts in volume are summarized below. Observations by Pryor, Hickam, Sieker and Page (14) and by Knowles, Hong and Rahn (13) suggest that an artifact in esophageal pressure is produced by the weight of heart and mediastinum upon the balloon catheter in the supine body position. This artifact was absent in the prone position. For this reason the supine position [used, incidently, by Marshall, McIlroy and Christie (4) in their study of several orthopneic patients] was avoided, and subjects were studied in only the prone and sitting positions. In two patients and two normal subjects compliance was studied repeatedly while resting in the supine position over a period of four hours. In these studies it was assumed that the esophageal pressure artifact would remain relatively constant and that therefore compliance changes occurring over a period of time represented changes in the properties of the lungs. In justification of this assumption were the observations that: *1*) No significant compliance changes were found in two normal subjects after four hours in the supine position, and *2*) no significant compliance changes were found in the two cardiac patients *after* successful treatment of their congestive failure though significant changes had occurred when these patients had first been studied prior to treatment.

In the studies of the acute effect of position change, volume-pressure measurements were made in the sitting position and within one to five minutes after changing to the prone position (as soon after the change as satisfactory measurements could be made). The eight normal subjects were all young male physicians or laboratory personnel without history or physical evidence of lung or heart disease. Of the eight patients with heart disease, four had rheumatic heart disease with predominant mitral valve involvement, and four had arteriosclerotic heart disease. At the time of study all patients had exertional dyspnea, orthopnea and crepitant rales confined to the bases of one or both lungs. It is recognized that the distinction between pulmonary edema of mild degree and "pulmonary congestion" is an arbitrary and purely clinical one. Right ventricular failure was also present in all with peripheral edema and elevated systemic venous pressure. A brief summary of each patient is presented in an appendix. In two patients four-hour supine

studies were done while in failure and repeated following successful treatment of failure; in one of these (Case 8, D.C.) no study of the acute sitting to prone compliance change was made. In two patients, studies of acute changes in compliance between sitting and prone positions were also repeated following recovery (Figure 4 and bottom of Table I).

RESULTS

Acute compliance changes in position change

Lung compliance did not change significantly in seven of the eight normal subjects studied (Table I) on changing from sitting to prone. In the normal subject in whom a significant change was seen, the compliance unaccountably rose by 38 per cent. In six of seven patients in whom the acute changes in compliance were studied on changing from sitting to prone, significant decreases in compliance were found. In the remaining patient compliance fell by 7 per cent, a decrease not statistically significant at the 5 per cent level. Case 4 was clinically in about the same degree of failure when studied on two occasions eight months apart. Compliance changes of similar magnitude were observed on these two occasions. The average fall in compliance in the patients with heart failure was 21.9 per cent. In Patients 5 and 6 the study of acute compliance changes was repeated after successful treatment of their failure two weeks and six months, respectively, after the first study. Whereas in the first study significant decreases in compliance (-32 and -24 per cent) were found, in the second study compliance did not change significantly in one and showed a slight but significant rise in the other from the sitting to the prone position (Figure 4). Of significance also is the observation that compliance returned to the sitting value in the five patients in whom measurements in the sitting position were repeated immediately after the prone determinations.

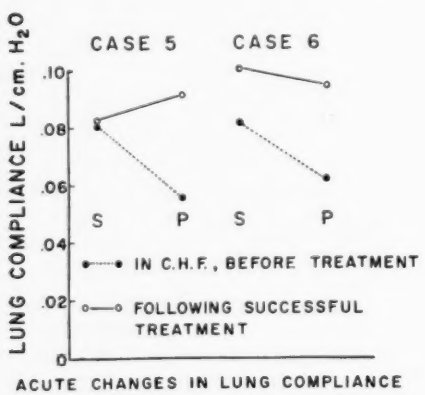

FIG. 4. ACUTE CHANGES IN LUNG COMPLIANCE WHICH OCCURRED ON CHANGE FROM THE SITTING TO THE PRONE POSITION IN TWO PATIENTS WITH CONGESTIVE HEART FAILURE

Solid points with dashed lines indicate change in compliance which occurred on changing from sitting to prone position prior to treatment; open points with solid line indicate change in lung compliance which occurred on position change following recovery from failure. In both cases the compliance decrease occurring while in failure was significant at the 1 per cent level. Following recovery a slight rise, significant at the 5 per cent level, was seen in Case 5 and in Case 6 a slight fall was seen which was not significant at the 5 per cent level.

Volume changes

In four of the normal subjects and four of the patients with heart failure, the vital capacity was measured with relation to the resting end-expiratory position in sitting and prone positions. In the four normal subjects vital capacity was 4.8 L. sitting and 4.4 L. in the prone position. Vital capacity consistently fell in changing from the sitting to the prone position, the change varying from 5.7 to 13.6 per cent and averaging 9.6 per cent. In the patients with congestive heart failure vital capacity averaged 2.04 L. sitting and 1.84 L. prone. In these patients the vital capacity also fell consistently in changing from the sitting to the prone position, the average change being 11 per cent with values ranging from minus 4 per cent to minus 16 per cent. In changing from sitting to prone the resting end-expiratory volume within the lungs fell, resulting in a decrease in the expiratory reserve volume. In the normal subjects, the decrease averaged 31 per cent. The decrease in expiratory reserve volume in the patients with heart failure ranged from 5 to 42 per cent with an average of 24 per cent. These changes in resting end-expiratory position were not measured directly but are inferred from changes in the expiratory reserve volume (ERV). Attempts were made to determine the shift in resting end-expiratory position directly by recording tidal volume continuously for several minutes

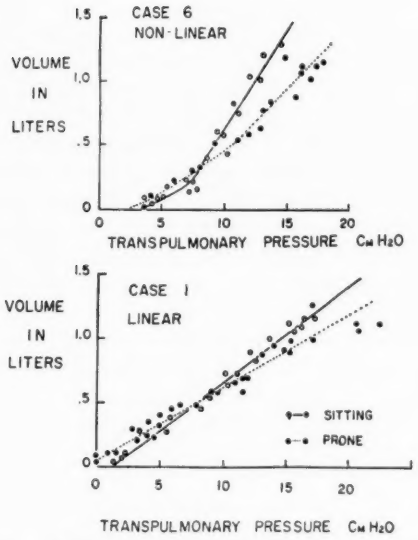

FIG. 5. INSPIRATORY VOLUME-PRESSURE CURVES IN CASES 6 AND 1 SHOWING EXAMPLES OF NONLINEAR AND LINEAR INSPIRATORY VOLUME-PRESSURE CURVES

The solid lines indicate the volume-pressure curves obtained in the sitting position; the dashed lines indicate the volume-pressure curves obtained in the prone position in each case.

before, during and after the position change. However, alterations in breathing pattern and in gas exchange coincident with the position change made this shift difficult to determine with accuracy. Much greater variation occurred upon repetition of these direct determinations than occurred among repeated indirect determinations obtained by subtracting the prone ERV from the sitting ERV.

Aside from the striking differences in vital capacity between the normal subjects and the patients with heart failure there were no significant differences in the volume data between the two groups when expressed on a percentage basis. There was no correlation between compliance change and volume change in either normal or heart failure groups in going from the sitting to the prone position.

Shape of volume-pressure curves

The inspiratory limbs of the lung volume-pressure curves were linear in Cases 1, 2, 4 and 7 and also in Case 8, in whom only supine studies were done. Cases 3, 5 and 6 showed nonlinear inspiratory volume-pressure curves with a flatter slope (lower compliance) at smaller lung volumes and steeper slope (higher compliance) as the lung expanded. Figure 5 illustrates typical linear and nonlinear inspiratory volume-pressure curves with the change occurring on shifting from the sitting to the prone position. It should be noted that merely shifting the resting end-expiratory position (volume) without any change in the slope of the volume-pressure curve could alone cause a fall in compliance in those cases where the volume-pressure curve was nonlinear. It is also of interest that in Case 5, the volume-pressure curve was nonlinear when the patient was first studied (in heart failure) but was linear two weeks later when the patient was restudied after successful treatment.

"Static" lung hysteresis was noted in most of the patients with heart failure and for comparably-sized breaths was greater than normal (15). It decreased but still remained greater than normal on recovery from heart failure. Figure 6 compares static volume-pressure curves showing hysteresis from Case 6 before and after treatment. A volume-pressure curve from a normal subject with the usual minimal degree of hysteresis is shown for comparison. Volume-pressure hysteresis was not appreciably different in the sitting and prone positions.

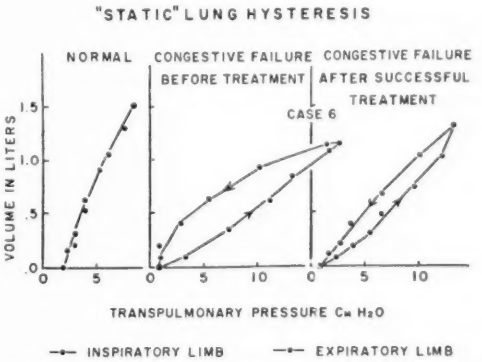

FIG. 6. "STATIC" LUNG VOLUME-PRESSURE CURVES MADE ON BREATHS OF COMPARABLE SIZE ON A NORMAL SUBJECT AND ON A PATIENT WITH CONGESTIVE FAILURE BEFORE AND AFTER SUCCESSFUL TREATMENT

The hysteresis "loop" is well shown; it is seen to be least in the normal subject and greatest in the patient with congestive failure prior to treatment.

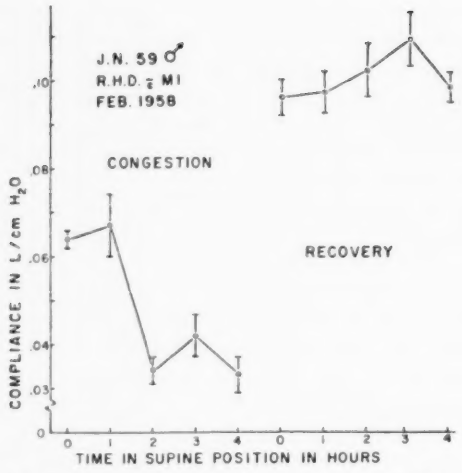

FIG. 7. COMPLIANCE CHANGE IN PATIENT 5 OCCURRING OVER TWO FOUR HOUR PERIODS OF OBSERVATION IN THE SUPINE POSITION

The second (recovery) study was done three weeks after the first. At the time of the first set of observations (labeled "congestion") the patient was in frank cardiac failure with edema and systemic venous hypertension. At the time of the repeat study (labeled "recovery") venous pressure was normal, edema was absent and patient had lost 45 pounds. The vertical brackets about each point indicate plus or minus two standard errors.

Prolonged studies in the supine position

In Patients 5 and 8 compliance was repeatedly measured while they rested in the supine position. Significant decreases in lung compliance were noted over four hour periods in both patients. These changes are shown in Figures 7 and 8. In spite of the considerable changes in compliance neither subject complained of increased breathlessness except when rebreathing from the spirometer, and there were no changes in physical findings over the chest to suggest the occurrence of clinical pulmonary edema. These two patients were restudied, Subject 5 three weeks after his first study, and Subject 8 one week after the initial study, on which occasions both patients were free of edema and systemic venous hypertension. The right-hand portions of Figures 7 and 8 labeled "recovery" show that no significant changes occurred during the repeat studies. Repeated measurements of compliance in two normal subjects over periods of four hours in the supine position showed no significant changes.

DISCUSSION

The acute decrease in lung compliance in cardiacs upon assumption of the prone position were of moderate degree ranging from 7 to 31.8 per cent. The absence of this change in normal subjects and its disappearance in the two patients restudied after successful treatment indicate that the changes are associated with the congested state and not due to an artifact of esophageal pressure registration as is known to occur in a supine position. The rapidity of this compliance fall (within one to five minutes) coupled with the observation made in five patients that the compliance fall was rapidly reversible argue against the change being due to pulmonary edema. Borst and co-workers (16) found in open-chest dog preparations that increases in pulmonary blood flow and pulmonary artery pressure produced no change in compliance so long as left atrial pressure (and inferentially pulmonary capillary pressure) remained normal. Acute and reversible decreases in compliance of 20 to 30 per cent were seen when left atrial pressure was elevated from normal levels (0 to 10 cm. H_2O) to 50 to 60 cm. H_2O. From their data the relationship between compliance and left atrial pressure, though inverse, appeared linear. The conclusion that pulmonary capillary pressure, probably because of its rela-

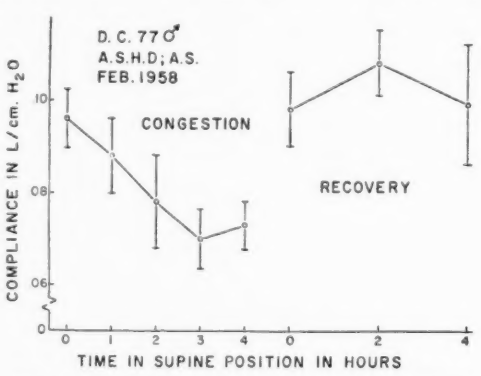

FIG. 8. COMPLIANCE CHANGE IN PATIENT 8 OCCURRING OVER TWO FOUR HOUR PERIODS OF OBSERVATION IN THE SUPINE POSITION

The second study was done one week after the first. When first studied (data shown under "congestion"), venous pressure was elevated and edema present. When restudied one week later he had lost 30 pounds, had normal venous pressure and no edema. The vertical brackets indicate plus or minus two standard errors.

tionship to pulmonary vascular volume, is an important determinant of lung compliance seems justified and worthy of emphasis.

It has been found in normal subjects by Sjöstrand (17) using plethysmographic techniques that an average of 640 ml. of fluid shifts from the lower portions of the body to the thorax on change from the erect to the recumbent position. Three-quarters of this volume was estimated to enter the lungs. Lägerlöf, Eliasch, Werkö and Berglund (18) found in one normal subject and one hypertensive patient that pulmonary capillary pressure and central blood volume rose on change from the upright to the horizontal position. Neither of these patients was in congestive heart failure, however. The lack of analogous data from patients with heart failure allows little more than speculation as to whether greater than normal increases in these variables occur in cardiacs in failure on assuming the horizontal position. Suggestive data on this point have however been provided by Donald, Bishop, Cumming and Wade (19). These investigators, studying the effects of position change in normals and cardiacs, found that pulmonary artery pressure rose to a greater extent in cardiacs than in normals in assuming the recumbent position. It was not determined whether this rise in pulmonary artery pressure was associated with a rise in pulmonary vascular resistance or with a rise in pulmonary capillary pressure.

The observations of increased pulmonary vascular volume and capillary pressure in normal subjects together with the observation that vital capacity decreases slightly on assuming the recumbent position (20) (a decrease, incidently, prevented by venous occlusion cuffs on the extremities) suggest that compliance should be slightly lower in normals in the recumbent position. The normal subjects in this study did not consistently show this. It is thought that the compliance fall in normals is so small as to be obscured by random measurement errors such as superimposed cardiac pulsations in the esophageal pressure records.

Evidence exists which indicates that in the chronically congested lung a large decrease in lung compliance may be produced by a relatively small increment in pulmonary capillary pressure. Saxton, Rabinowitz, Dexter and Haynes (6) found reversible decreases in compliance to one-half the resting value in cardiacs on exercise. These large decreases in lung compliance were in some cases associated with pulmonary capillary pressure rises as small as 6 to 10 mm. Hg.

Left for future investigations to explore are the questions:

1. Does a greater increase in pulmonary vascular volume and capillary pressure occur in the cardiac in failure than in the normal on change from the upright to the recumbent?

2. What is the difference between normal and chronically congested lungs with respect to the relationship between vascular pressure and volumes and lung compliance?

The slower changes in compliance which occurred in Patients 5 and 8 when they were studied repeatedly in the supine position may be due to mild degrees of pulmonary edema. It is of interest that in Case 5 a fall in compliance of 50 per cent was associated with no increase of either physical signs or respiratory distress so long as the patient was allowed to lie quietly. Changes in the shape of the volume-pressure curve in this patient indicating extreme stiffness at the beginning of inspiration are similar to observations made in cases of pulmonary edema (5), and suggest its presence. In Case 8, however, volume-pressure curves were linear, changing only their slope but not their shape over the period of supine study. The compliance changes in this case may be on the basis of a gradual rise in pulmonary capillary pressure over the period of study. Unfortunately the obvious experiment of returning these patients to the sitting position and ascertaining the time necessary for compliance to return to the former sitting level was not done.

The curvilinearity of inspiratory volume-pressure curves in several of the patients and the observation of increased static hysteresis suggest exclusion of some groups of alveoli at lower lung volumes which inflate only when their opening pressures are exceeded. Whether collapse is at a bronchiolar level which would result in air trapping or whether there is true collapse of alveoli as observed by Radford and McLaughlin (21) cannot be determined on the basis of the data presented.

The relationship of the observations presented to orthopnea deserves a few words. In this re-

port, the lungs of several orthopneic patients have been observed to become physically stiffer when these patients assumed a horizontal position. This implies rather directly that the work required to ventilate the lungs of these patients was greater in the horizontal than in the upright position. Though one cannot equate increase in respiratory work with an onset of or increase in dyspnea, there is undeniably a relationship between the two, and it is not unreasonable to suspect that the increased ventilatory work which these patients performed had something to do with the dyspnea which they described when they were horizontal. The subjective symptom orthopnea is probably the result of many factors, the exploration of which is beyond the scope of this discussion. The author suggests that the observations reported may be among the more important of these factors.

One final and fundamental point deserves comment relating to the tacit assumption of identity of esophageal and intrapleural pressures. That these pressures are not identical in the supine position has been previously noted (13, 14). In heart disease, where the left atrium, pulmonary veins and other structures close to the esophagus are distended, it may be that the esophageal pressure reflects abnormalities in these structures to a greater extent than alterations in the properties of the lung substance proper. Elucidation of this point awaits comparative studies of pleural and esophageal pressures in human disease states including congestive failure.

CONCLUSIONS

1. In seven patients with frank congestive heart failure lung compliance fell an average of 21.9 per cent when the body position was changed from sitting to prone; in six of the seven cases the decreases were significant at the 5 per cent level. These compliance decreases were rapid and were promptly reversible. In two of these cases repeat study following successful treatment no longer showed this compliance fall. Eight normal subjects similarly studied showed no significant fall in compliance.

2. In three of the seven patients the inspiratory volume-pressure curves of the lung were curvilinear with lower compliance at small inspired volumes. This change became more marked on assuming the prone position. In the remaining four patients the inspiratory volume-pressure curves were linear.

3. "Static" lung volume-pressure hysteresis was increased in pulmonary congestion and appeared to be roughly proportional to the degree of failure. Hysteresis was not significantly different in sitting and prone positions.

4. In two patients with congestive failure progressive decreases in compliance were noted over four hours when these patients were studied lying quietly in the supine position. These changes were absent on a later occasion when failure was no longer present. Furthermore, normal subjects similarly studied showed no change in compliance. In at least one of these cases the occurrence of sub-clinical pulmonary edema may be the explanation of the compliance fall.

5. It is suggested that the acute and chronic changes in lung compliance observed are partially responsible for the phenomenon of orthopnea.

APPENDIX

Case 1, T.D., a 35 year old white male, had rheumatic heart disease with stenosis of mitral and aortic valves. On the day prior to compliance studies, right and left heart catheterization had revealed a mean left atrial pressure of 27 mm. Hg, a pulmonary artery pressure of 65/33, and gradients of 12 and 70 mm. Hg, respectively, across the mitral and aortic valves. The cardiac index was 2.5 L. per minute per $M.^2$. Right atrial pressure was 10 mm. Hg. Peripheral edema and elevated venous pressure were noted clinically on the day of study.

Case 2, W.F., was a 28 year old white male with rheumatic heart disease and mitral stenosis who had undergone two commissurotomies, both unsuccessful because of extensive valve calcification. On the day of study the venous pressure was above 180 mm. of H_2O and edema was present to the level of the knees.

Case 3, F.G., was a 46 year old white male with rheumatic heart disease and mitral stenosis who had not improved following a commissurotomy two years before. On the day of study venous pressure was above 180 mm. of H_2O. Peripheral edema was present and the liver was enlarged and tender.

Case 4, C.M., was a 58 year old white male with arteriosclerotic heart disease. On the day of study the venous pressure was 100 mm. of H_2O. Marked edema was present and his weight was 200 pounds. When restudied seven months later the venous pressure was 80 mm. of H_2O and his weight was 197 pounds. Moderate edema persisted to the level of mid-tibia.

Case 5, J.N., was a 60 year old Negro male with rheumatic heart disease with predominant mitral insufficiency. On the day first studied, venous pressure was 180 mm.

of H_2O, edema was present to the level of mid-thigh, the liver was enlarged and he weighed 200 pounds. When restudied three weeks later his weight was 155 pounds, venous pressure was normal and edema, absent.

Case 6, W.W., was a 41 year old Negro male with rheumatic heart disease with predominant mitral stenosis. At the time of the first study the venous pressure was clinically elevated, peripheral edema was present, and the liver was enlarged and tender. Cardiac catheterization done one week before the study revealed a mean left atrial pressure of 25 mm. Hg and a pulmonary artery pressure of 91/35 at rest. When restudied eight months later and seven months following a successful mitral commissurotomy, the venous pressure was normal, no edema was present, and the patient had nearly normal exercise tolerance.

Case 7, T.J., was a 78 year old diabetic woman with arteriosclerotic heart disease. On the day studied the venous pressure was clinically elevated, peripheral edema was present and the liver was enlarged and slightly tender.

Case 8, D.C., was a 77 year old white male with arteriosclerotic heart disease. When first studied the venous pressure was clinically elevated, edema was present to the level of mid-thigh and the liver was enlarged and tender. His weight was 180 pounds. When restudied one week later venous pressure was clinically normal, edema was absent, the liver smaller and the weight 150 pounds. Supine studies only were done on this patient.

Except in Case 2, venous pressure was estimated from the neck veins by the method of Sir Thomas Lewis (22), and where numerical values are given, the reference level is the sternal angle.

ACKNOWLEDGMENTS

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STUDIES ON CARBOHYDRATE METABOLISM IN PATIENTS WITH NEOPLASTIC DISEASE. II. REPONSE TO INSULIN ADMINISTRATION *†

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A decrease in glucose tolerance, as indicated by a slower rate of disappearance of blood glucose following intravenous glucose administration, has been observed in patients with neoplastic disease (2). The group studied included subjects with chronic diffuse lymphomas and leukemias as well as clinically localized carcinomas who were carefully selected to exclude various other factors known to influence carbohydrate utilization. In view of the fact that the altered glucose metabolism was associated with localized carcinoma, it appeared likely that it reflected a defect in the metabolism of the host. The mechanism of this defect is unknown.

In view of suggestions (3, 4) that endogenous insulin secretion determines the rate of blood glucose disappearance, the decreased glucose tolerance might reflect either diminished secretion of, or a diminished responsiveness to, endogenous insulin. On the assumption that sensitivity to exogenously administered insulin reflects responsiveness to the endogenously secreted hormone, insulin response tests have been performed in patients with neoplastic disease. This has been done employing a three hour test procedure which permitted the determination of the rate constant of the decline in blood glucose concentration, first, following the intravenous administration of glucose sufficient to cause hyperglycemia, and second, following the intravenous administration of insulin. It was found that patients with neoplastic disease, compared to control subjects, had an impaired response to insulin as well as a decreased glucose tolerance.

METHODS

Subjects studied. The control group consisted of 16 individuals without neoplastic disease, eight males and eight females, ranging in age from 36 to 61 years. The patients with neoplastic disease included 24 individuals, 11 males and 13 females, ranging in age from 33 to 71 years. There were no significant differences between the control and the patient groups with respect to age or body weight. The diagnoses of these subjects are indicated in Tables I and II. Criteria (2) were used in selection of the subjects for this study which insured, as far as was possible on a clinical basis, that tests were performed only on persons in whom various factors known to impair carbohydrate metabolism could be excluded.

Procedures. All subjects were placed on a daily diet containing at least 225 Gm. of carbohydrate and 2,400 calories for more than one week prior to study. Each subject, following an overnight fast, rested in an air-conditioned room for one-half hour prior to and during the test. An indwelling, Cournand-type needle was placed in an antecubital vein. After two fasting blood samples had been obtained over a period of 20 to 30 minutes, approximately 25 Gm. of glucose (as a 30 per cent solution in distilled water) was injected into another vein over a four minute period. Following the completion of the glucose injection venous blood samples were withdrawn without stasis into heparinized syringes at eight minute intervals for 64 minutes. At approximately 75 minutes following the glucose injection, another sample of blood was obtained and a standard dose of glucagon-free insulin[1] (0.1 unit per Kg. body weight) was administered intravenously through the indwelling needle. Seven venous blood samples were obtained at three minute intervals between seven and 25 minutes following the insulin injection. Then the test was terminated by the intravenous administration of 30 ml. of a 30 per cent glucose solution and the oral administration of sweetened orange juice. The development of hypoglycemic symptoms necessitated earlier termination of the procedure in only two subjects.

* A portion of the present data was presented before the American Association for Cancer Research, April, 1957, and appears in abstract form (1).

† This work was supported in part by Grant C2332 of the National Cancer Institute.

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[1] Glucagon-free insulin was kindly supplied by The Eli Lilly Co., Indianapolis, Ind.

TABLE I

Response to intravenous administration of glucose followed by insulin in control subjects

Subject number*	Blood glucose concentration		Rate constants of decline in blood glucose concentration§		
	Fasting†	Before insulin‡	k_G	k_I	k_I/k_G
	mg. %	*mg. %*	*%/min.*	*%/min.*	
1	85–88	73	1.31	4.62	3.53
2	78–80	76	1.58	9.90	6.27
3	83–81	64	1.88	6.30	3.35
4	81–72	104	1.59	6.90	4.34
5	92–88	131	1.61	4.78	2.97
6	76–77	104	1.26	5.92	4.70
7	83–85	84	2.15	10.30	4.79
8	88–86	88	1.92	6.30	3.28
9	80–82	114	1.87	7.42	3.97
10	86–90	104	1.84	8.00	4.35
11	87–88	100	1.69	6.12	3.62
12	83–90	106	2.21	9.30	4.21
13	78–82	91	1.98	7.04	3.56
14	82–85	97	1.82	6.42	3.53
15	83–86	95	1.89	6.00	3.17
16	81–85	94	1.60	5.00	3.13
Average	83	95	1.76	6.90	3.92
S. D.	4	16	0.26	1.86	0.82

* Diagnoses: Subjects 1 and 3, benign breast adenoma; Subject 2, bronchopneumonia, convalescent; Subject 10, cervical erosion; Subjects 13 and 14, tuberculoma of the lung; Subjects 4–9, 11, 12, 15, 16, normal volunteers.

† Two fasting blood sugar determinations were performed approximately 20 minutes apart.

‡ This blood sugar determination was performed approximately 75 minutes following glucose administration and immediately prior to insulin injection.

§ k_G represents the rate constant following glucose administration; k_I represents the rate constant following insulin administration.

Whole blood glucose concentrations were determined in duplicate by the Nelson modification of the Somogyi method (5, 6).

Analysis of the responses to glucose and to insulin administration. The responses to glucose and to insulin administration were analyzed by graphing the logarithm of the total blood glucose concentration against time (7). It was observed that the blood glucose concentration decreased at an exponential rate during the mid-portion of the period following both glucose and insulin administration. This linear function persisted for at least 40 minutes after glucose administration and at least 12 minutes after insulin administration. It is assumed that during this period of exponential decline of the total blood glucose concentration the slope of the plotted curve provides an index of the response to either glucose or insulin administration. This index may be expressed as the rate constant for the assumed first-order reaction according to the equation:

$$k_G \text{ or } k_I = \frac{\log_e 2}{t_{\frac{1}{2}}},$$

where k_G and k_I are the fractional rate constants of disappearance of blood glucose following glucose or following insulin administration, respectively, and $t_{1/2}$ is the time in minutes for the total blood glucose concentration to decline 50 per cent from any point after glucose or after insulin administration, respectively. An example of this graphic analysis is presented in Figure 1.

It has been previously demonstrated (2) that this empirical method of analysis of intravenous glucose tolerance curves provides good reproducibility in a given subject, and good discrimination between the control and neoplastic groups.

RESULTS

The data obtained from the tests of the response to glucose and to insulin administration in 16 control subjects and 24 patients with neoplastic disease are summarized in Tables I and II. The rate constant for the decline in blood glucose concentration after insulin administration (k_I) for the control group was 6.90 ± 1.86 per cent per minute [2] compared to 3.99 ± 1.30 per cent per minute in the patient group. The similar rate constant after glucose administration (k_G) was 1.76 ± 0.25 per cent per minute for the control group and 1.18 ± 0.37 per cent per minute for the patient group. The differences between the means of both k_I and k_G were statistically significant, 5.5 and 5.9 times the standard error of the difference, respectively. The values for k_G in the control and patient groups are in agreement with previous

[2] Average values are given with one standard deviation.

studies of a larger number of subjects (2). The ratio of k_I/k_G for the control and the patient groups were 3.92 ± 0.82 and 3.46 ± 0.91, respectively. These ratios are not significantly different.

The fasting blood glucose concentration averaged 83 ± 4 mg. per cent in the control group and 85 ± 10 mg. per cent in the patient group. The difference between these means is not statistically significant. The blood glucose concentration prior to the administration of insulin did not differ significantly in the two groups (95 ± 16 mg. per cent and 109 ± 25 mg. per cent in the control and patient groups, respectively). Nevertheless there was a tendency for the glucose concentration to be higher in the patient group than in the control at the time of insulin administration. Accordingly, the effect of varying concentrations of blood glucose at the time of insulin administration upon k_I was studied in three patients with neoplastic disease and in three control subjects (Table III). In these six subjects, k_I differed slightly, if at all, on repeat studies, despite differences in the pre-insulin administration blood glucose concentrations of 25 to 65 mg. per cent.

DISCUSSION

A decreased sensitivity to insulin, as indicated by a slower rate of decline in blood glucose concentration following the intravenous administration of this hormone, was observed in patients with neoplastic disease. These subjects also demonstrated impaired glucose tolerance, as previously described (2, 8–13).

In terms of the rate of disappearance of blood glucose, the patients with neoplastic disease ex-

TABLE II

Response to intravenous administration of glucose followed by insulin in patients with neoplastic disease

Subject number*	Blood glucose concentration		Rate constants of decline in blood glucose concentration§		
	Fasting†	Before insulin‡	k_G	k_I	k_I/k_G
	mg. %	*mg. %*	*%/min.*	*%/min.*	
17	98–93	150	0.90	3.96	4.40
18	84–81	106	1.33	5.90	4.44
19	80–81	128	1.17	4.34	3.71
20	80–84	93	1.17	3.07	2.62
21	88–87	89	1.24	3.98	3.21
22	119–115	178	0.94	2.47	2.63
23	80–85	116	1.25	4.60	3.68
24	98–94	108	1.12	3.66	3.27
25	89–87	84	1.80	4.84	2.69
26	89–84	96	1.21	2.95	2.44
27	86–84	147	1.05	4.77	4.54
28	85–88	100	1.21	2.89	2.39
29	82–86	90	0.90	2.57	2.86
30	78–74	80	2.36	7.80	3.31
31	82–73	80	0.93	2.28	2.45
32	75–78	94	1.82	4.62	2.54
33	75–74	84	1.41	4.47	3.17
34	87–89	114	0.75	2.16	2.88
35	91–91	92	1.10	6.00	5.45
36	88–93	108	1.07	3.19	2.98
37	54–63	127	0.90	3.85	4.28
38	77–75	90	1.16	4.46	3.84
39	87–89	116	1.05	3.85	3.67
40	84–84	150	0.53	2.99	5.64
Average	85	109	1.18	3.99	3.46
S. D.	10	25	0.37	1.30	0.91

* Diagnoses: Cases 17–21, carcinoma of the cervix, clinical stage I or II; Cases 22–24, carcinoma of the breast; Cases 25–29, Hodgkins disease; Cases 30 and 31, reticulum cell sarcoma; Cases 32–36, lymphosarcoma; Case 37, chronic lymphatic leukemia; Cases 38 and 39, chronic myelogenous leukemia; Case 40, giant follicular lymphosarcoma.

† Two fasting blood sugar determinations were performed approximately 20 minutes apart.

‡ This blood sugar determination was performed approximately 75 minutes following glucose administration and immediately prior to insulin injection.

§ k_G represents the rate constant following glucose administration; k_I represents the rate constant following insulin administration.

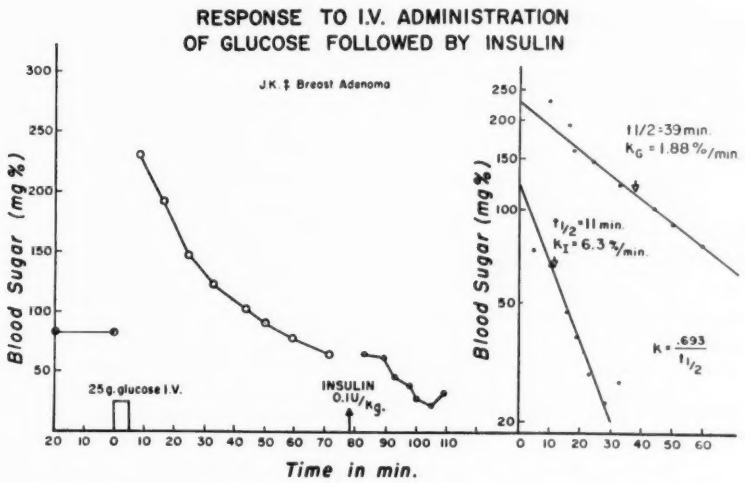

FIG. 1. RESPONSE TO I.V. ADMINISTRATION OF GLUCOSE FOLLOWED BY INSULIN

The left-hand portion of this figure presents the blood glucose concentration data after intravenous administration of glucose followed by insulin in control Subject J.K. The right-hand portion of this figure presents the graphic analysis of these data to obtain the rate constants of glucose disappearance following administration of glucose (k_G) and insulin (K_I). $T_{1/2}$ is the time in minutes for the total blood glucose concentration to decline 50 per cent from any point after glucose or insulin injection.

hibited the same relative degree of unresponsiveness to exogenous insulin as to intravenously administered glucose. This is indicated by the finding that the ratio of the rate constants, k_I/k_G, did not differ significantly in the patient and control groups. In view of the evidence that the rate of blood glucose disappearance during hyperglycemia may be determined by endogenous insulin secretion (3, 4), these observations are compatible with the concept that impaired glucose tolerance in patients with neoplastic disease may be a manifestation of a decreased responsiveness to endogenous insulin.

It is to be stressed that a decrease in reponsiveness to insulin is not specific. It has been described in a variety of disorders (14, 15), including diabetes mellitus, endocrine dysfunctions, hepatic disease and infectious diseases and in association with increasing age (16).

The present study indicates that decreased in-

TABLE III

The effect of different concentrations of blood glucose on the response to insulin administration

Patients with neoplastic disease			Control subjects		
Subject no.	Blood glucose concentration before insulin*	Rate constant k_I	Subject no.	Blood glucose concentration before insulin†	Rate constant k_I
	mg. %	*%/min.*		*mg. %*	*%/min.*
33	84	4.47	4	104	6.90
	120	4.50		159	6.50
37	127	3.85	10	104	8.00
	151	3.70		131	8.54
38	90	4.46	16	94	5.00
	118	4.68		158	4.74

* Insulin was administered to the patients 75 minutes following glucose administration.
† Insulin was administered to the control subjects 40 minutes following glucose administration.

sulin responsiveness is a manifestation of the metabolic alterations associated with neoplasia in the human host, but provides no data which permit conclusions as to the mechanism of this alteration in hormonal control.

SUMMARY

1. A procedure for testing the response to intravenous glucose administration as well as the response to intravenous insulin administration has been employed which permits their comparison by means of the rate constant of the decline in blood glucose concentration. This procedure is performed in a given subject during a single three hour study.

2. An impaired response to insulin administration as well as the previously described impairment in glucose tolerance was observed in 24 patients with neoplastic disease, when compared with 16 control subjects.

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THE GRANULOCYTOPENIC RESPONSE IN HEMORRHAGIC SHOCK *

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When endotoxins are given intravenously the granulocytes leave the active stream of the circulation and congregate chiefly in the capillaries of the lung, spleen and liver. The same phenomenon can be expected to occur in hemorrhagic shock because experimental evidence suggests that the loss of the antibacterial defenses in shock results in the development of an endotoxemia (1). This paper presents the results of a comparative study of the circulating granulocytes in hemorrhagic shock and in endotoxemia, including data concerning the possible mechanisms involved.

METHOD

White adult albino rabbits and mongrel dogs were employed in this study. Endotoxemia was induced by injecting an *Escherichia coli* endotoxin (Difco Batch No. 0127-B8) intravenously. In rabbits this endotoxin was found to have an MLD per 100 of 2 mg. per Kg. and an MSD per 100 (MSD = maximal surviving dose, *i.e.*, the largest dose resulting in a zero mortality) of 0.05 mg. per Kg. These toxicity values were verified in rabbits prior to each experiment. Dogs were tolerant to at least 10 rabbit MLD's per 100 of this endotoxin. Hemorrhagic shock was induced as described elsewhere (2).

Total and differential counts of the blood were made in the normal state, and at different intervals after inducing shock or endotoxemia, beginning as early as one minute after challenge, again after 15, 30 or 60 minutes, and again every hour for several hours. Occasionally in survivors the count was obtained 24 hours later. The counts were made on blood from a large vein or artery according to convenience. There was no significant difference in the counts of venous and arterial blood.

Leukocyte counts were made in a variety of circumstances as follows:

I. Effect of variation of dose of endotoxin in normal rabbits. The response to four different doses of endotoxin (0.0002, 0.0004, 0.8 and 1.5 of one MLD per 100) was observed in four groups of four or more rabbits each. The purpose of this range of dosage was to observe the severity and duration of the granulocytopenia in response to a very light and a very severe injury.

II. Effect of tolerance to endotoxin. Tolerance was induced in normal rabbits by a daily intravenous injection of 0.07 MLD per 100 for seven successive days. Tolerance signifies an enhanced capacity to detoxify endotoxin. If the flight of the granulocytes is a direct effect of unneutralized endotoxin one might expect the granulocyte count to drop less severely or not at all in the tolerant animal; and if a drop occurs, that return to a normal count should occur more rapidly than in the nontolerant animal. Counts were also made each day before and after injection of the endotoxin in order to observe any change in response as tolerance develops.

III. Effect of hemorrhagic shock. Transfusion for shock of one and one-half hours' duration usually results in recovery (reversible shock). Transfusion for shock of four to six hours' duration is usually futile and death follows (irreversible shock). If the thesis is correct that irreversibility is due to the accumulation of a lethal dose of toxin, the curve of the granulocyte count for reversible and irreversible shock might resemble the respective curves for a sublethal and a lethal dose of toxin. Accordingly, hemorrhagic shock was produced and counts were taken before inducing shock, and at intervals before and after transfusion for shock of one and one-half hours' duration, and for shock of six hours' duration.

IV. Effect of plasma from irreversibly shocked rabbits in normal recipients. If shock plasma contains endotoxin, the granulocyte response in a normal recipient of such plasma (10 to 12 ml.) should resemble that in response to injected endotoxin. The extent and duration of the granulocytopenia might be a rough measure of the amount and toxicity of the endotoxin in the shock plasma.

The plasma was obtained as follows: Rabbits in hemorrhagic shock produced by the technique described (2) were transfused with all of their own shed blood after six hours, and when the resulting pressor response failed the rabbit was exsanguinated. The heparanized blood collected in sterile containers was promptly centrifuged, the plasma tested at once, or stored immediately thereafter in sterile containers at 4° C., and tested later. (Storage at this temperature for days or weeks does not reduce the initial toxicity of such plasma.)

V. Effect of hemorrhagic shock pretreated with nonabsorbable antibiotics. As already reported, such pre-

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† Holder of Traveling Fellowship from the Leeds General Infirmary, Leeds, England.

treatment prevents the plasma of the animal in shock from becoming toxic to a vulnerable recipient (1). The granulocyte response to such plasma should differ from the response to plasma from shocked animals not given antibiotics because of the presumed absence of endotoxin, and should reveal any effect of the shock process on the granulocyte count which is not due to endotoxin.

The antibiotics used were Bacitracin® (100,000 units) and polymyxin (100 mg.) given to rabbits in water by gavage daily for five successive days. At this time stool cultures were free of all flora except small numbers of *B. Proteus* and *Pseudomonas*. Several hours after the fifth dose hemorrhagic shock was induced and continued for six hours, after which the animal was transfused with its own shed blood.

VI. Effect of epinephrine. Immediate and severe vasoconstriction is produced by hemorrhagic shock or by endotoxin. Since there is reason to believe that endotoxin in the normal and shocked animal exerts its adverse effects on the peripheral vessels via epinephrine (3), the role of epinephrine in the granulocyte response was studied in two ways: *1*) by observing the effect of an intravenous injection of epinephrine (in normal rabbits 0.1 ml. of 1:100,000 dilution and in normal dogs, 0.8 ml. of 1:1,000 dilution), or of norepinephrine (1 ml. of 0.2 per cent solution); and *2*) by the use of adrenergic blockade. The latter approach is more appropriate to the problem because, if epinephrine plays a role in the granulocyte response in hemorrhagic shock or endotoxemia, it is presumably acting more or less continuously. But because a satisfactory method for continuous and prolonged administration of epinephrine that the rabbit will tolerate is not yet available, the role of this substance acting continuously should be disclosed inferentially by the response to dibenamine. Dibenamine was given to rabbits (20 mg. per Kg.) two to three hours prior to a dose of endotoxin (1.2 MLD per 100 or a 0.0004 MLD per 1000), and prior to hemorrhagic shock terminated by transfusion after one and one-half hours.

RESULTS (TABLES I THROUGH VI)[1]

The total count and the granulocyte count in any single observation are given as the mean values for the group. In no case were there less than four animals in a group. Changes in response to treatment are expressed in per cent of the pretreatment level.

Experiment 1—Effect of variation in dose of endotoxin

Table I demonstrates that in rabbits the granulocyte count falls to an extremely low level. This occurs almost immediately (one minute). After a lethal dose of endotoxin significant recovery from the fall does not occur at any time before death. The extent of the fall is less the smaller the dose, and the speed of recovery tends to be in

[1] The "approximate standard error" and "per cent of the pretreatment level" for each observation in the tables was calculated by Miss Rita Nickerson under the supervision of Dr. Mindel C. Sheps of the Department of Preventive Medicine of the Harvard Medical School. The procedure employed is described by her as follows:

Analysis of variance on the logarithms of the counts was performed on the data from each experiment separately. The sequential mean values shown in the table for each particular experiment are, therefore, the geometric means of the counts observed.

The standard errors of the mean log counts were calculated from the residual error term in each analysis of variance. The transformation into antilogarithms may be illustrated on the following values from the granulocyte counts observed in Experiment III, A on rabbits.

	Mean	S. E.	Mean ± 1 S. E.
At onset:			
Logs	3.726	0.108	3.618–3.834
Antilogs	5,321	1.284	4,150–6,830
At 1 hour:			
Logs	2.632	0.108	2.524–2.740
Antilogs	429	1.284	334–550

Since the calculations were done on the log counts the standard error of a mean can be stated precisely only in terms of logs. For presentation in terms of cell counts, "approximate standard errors" were computed in two ways:

a) From the antilogarithm of the standard error (1.284) as shown above, the per cent standard error is approximately 100(1.284 − 1) = 28 per cent (see Table III, A) (4).

b) In terms of cell counts themselves the "approximate standard error" of any mean in this experiment is 28 per cent of this mean. Therefore, at the onset the approximate standard error is (0.28) (5,321) = 1,490 cells and at one hour it is (0.28) (429) = 120 cells.

Each experiment consisted of repeated observations made on each of several animals. The experimental results, therefore, were considered not by comparing absolute counts, but by noting the logarithm of the per cent change as shown in the tables. To test the difference between the values at one hour, say, in two separate experiments, therefore, the following procedure was adopted:

	Mean log count at onset minus mean log count at 1 hour	(Standard error of difference)[2]
Rabbits' granulocytes Table III, A	1.094	0.0234
Rabbits' granulocytes Table III, C	0.448	0.0072
Difference in one hour values	0.646	0.0306

$$t = \frac{0.646}{\sqrt{0.0306}} = 3.69 \qquad p < 0.01$$

TABLE I

Effect of various doses of an E. coli endotoxin on total number of leukocytes and of circulating granulocytes in the normal adult rabbit (seven to eight animals in each group)

Endotoxin		Mean no. of granulocytes/ cu. mm.	Approximate S. E. (13%)	Per cent of pretreatment level	Mean of total white cells/ cu. mm.	Approximate S. E. (8%)	Per cent of pretreatment level
Dose of	Time						
	hrs.						
0.0002 MLD	0	5,309	690		8,830	706	
	1	1,523	198	29	4,943	395	56
	2	4,416	574	83	7,430	594	84
			(17%)			(8%)	
0.014 MLD	0	5,781	983		8,511	681	
	1	582	99	11	2,897	232	34
	2	649	110	11	2,577	206	30
	3	1,774	302	31	4,150	332	48
			(34%)			(14%)	
0.8 MLD	0	4,227	1,437		6,903	966	
	2	309	105	7.3	2,075	290	30
	4	505	172	12	2,697	378	39
			(38%)			(15%)	
1.5 MLD	0	2,685	1,020		6,040	906	
	¼	275	104	10	2,710	406	45
	1	121	46	4.5	2,109	316	44
	2	129	49	4.8	1,548	232	26
	3	140	53	5.2	1,664	250	28
	4	268	102	10	1,714	257	28

rough inverse proportion to the dose. In survivors an overshoot occurs in all instances within 24 hours, and often within six to eight hours after small doses.

The fall in the total count is due largely to the fall in the granulocytes. With all doses there is a transient absolute lymphocytosis, which disappears within an hour or two. Following large doses there may be a lymphocytopenia by the third or fourth hour.

In concurrent experiments on the rabbit, to be reported, the granulocytes leaving the circulation, as already noted by others (5, 6), were seen congregating chiefly in the capillaries of the lung.

TABLE II

Leukopenic response to 0.07 MLD E. coli endotoxin in nontolerant and tolerant rabbits (five animals in each group)

Time	Mean no. of granulocytes	Approximate S. E. (17%)	Per cent of pretreatment level	Mean of total white cells	Approximate S. E. (12%)	Per cent of pretreatment level
hrs.			Nontolerant			
0	5,420	921		8,129	975	48
½	1,242	211	23	3,945	473	21
1	389	66	7.2	1,698	204	32
2	361	61	6.7	1,812	260	22
3	468	80	8.6	1,762	211	22
4	908	154	17	1,941	233	24
5	1,689	287	37	3,296	396	40
6	3,837	652	71	6,886	826	85
			Tolerant			
		(14%)			(8%)	
0	9,730	1,362		13,303	1,064	
½	698	98	7.2	3,750	300	28
1	2,831	396	29	5,807	465	44
2	6,339	887	65	8,790	703	66

TABLE III

Leukopenic response to reversible and irreversible hemorrhagic shock in the dog and rabbit *

Time	Mean no. of granulocytes	Approx. S. E. 28% (23%)	Readings as % of pretreatment level	Mean total white cell count	Approx. S. E. 15% (17%)	Readings as % of pretreatment level
		A. Reversible shock†				
hrs.						
0	5,321 (7,585)	1,490 (1,744)		8,110 (10,422)	1,216 (1,771)	
1/6	1,247 (5,012)	349 (1,153)	23 (66)	4,842 (8,750)	726 (1,488)	60 (84)
1/2	457 (3,483)	128 (801)	8.6 (46)	3,589 (6,607)	538 (1,123)	44 (63)
1	429 (3,990)	120 (918)	8.1 (53)	3,614 (7,620)	542 (1,295)	45 (73)
4	2,162 (6,998)	605 (1,610)	41 (92)	6,576 (12,302)	986 (2,091)	81 (118)
		B. Irreversible shock‡				
		39% (18%)			13% (12%)	
0	4,169 (2,790)	1,626 (2,302)		7,551 (17,660)	982 (2,119)	
1/6	767 (5,544)	299 (998)	13 (43)	5,035 (9,955)	655 (1,195)	67 (56)
1/2	396 (4,074)	154 (733)	9.5 (32)	3,565 (8,200)	463 (991)	47 (47)
1	281 (5,755)	110 (1,036)	6.7 (45)	3,963 (10,715)	515 (1,286)	42 (61)
2	658 (5,285)	257 (951)	16 (41)	4,488 (9,185)	583 (1,102)	59 (52)
6	813 (6,290)	317 (1,246)	20 (54)	4,677 (18,115)	608 (2,174)	60 (103)
		C. 6 hour shock in endotoxin-tolerant rabbits (5 expts.)				
		15%			11%	
0	6,487	973		9,638	1,060	
1/2	2,260	339	35	4,634	510	48
1	2,312	347	36	5,035	554	52
2	3,614	542	56	6,486	713	67
3	5,152	773	79	8,280	911	86
6	5,597	840	86	8,830	971	92

* Figures in parentheses apply to dogs; figures not in parentheses apply to rabbits.
† Four dogs, eight rabbits.
‡ Six dogs, four rabbits.

liver and spleen. With the return of the total and granulocyte count toward normal, many of the circulating granulocytes in stained smears were young forms, and there was a noteworthy concurrent rise in the number of normoblasts. Hence new cells from the bone marrow replaced the segregated cells, many of which were still *in situ* 24 hours later, undergoing lysis, or ingestion by macrophages.

Dogs withstood 10 rabbit MLD's per 100 of the Difco endotoxin without noticeable effect, and without a fall in the total or granulocyte count. Larger doses were not tried.

Comment. Thus it appears that in rabbits, many, if not all, the granulocytes which leave the axial stream are rendered functionless permanently, presumably as a result of injury by the injected endotoxin. Failure of recovery of a normal count when the dose of endotoxin is lethal may mean suppression of bone marrow function by the endotoxin, or damage by circulating endotoxin to young granulocytes, and resultant segre-

TABLE IV

Leukopenic response of normal rabbits to plasma (10 to 12 ml.) from irreversibly shocked rabbits * (*five experiments*)

Time	Mean total of granulocytes	Approx. S. E. 36%	Readings as % of pretreatment level	Mean total of white cells	Approx. S. E. 12%	Readings as % of pretreatment level
hrs.						
0	5,224	1,881		8,204	984	
1	1,549	558	30	3,873	465	47
2	2,767	996	50	5,495	659	67
3	3,435	1,237	66	5,890	707	22

* Leukopenic response of normal rabbits to normal plasma is insignificant.

gation as rapidly as they are released into the circulation.

Experiment 2—Effect of tolerance to endotoxin

The fall in the total and granulocyte count immediately after administration of the endotoxin was about the same with each successive daily injection for seven days. But, as Table II shows, while the initial leukopenic response is the same in tolerant and nontolerant rabbits, the rate of return to normal increases with the acquisition of tolerance. After two hours the granulocyte count is some 90 per cent below the control value in the nontolerant rabbit, but it is only 35 per cent below in the tolerant rabbit.

Comment. The rate of delivery of granulocytes to the circulation from the bone marrow and the segregation of circulating granulocytes in the lungs, liver and spleen appears to be dependent on the speed of detoxification of the endotoxin.

Experiment 3—Leukopenic response to hemorrhagic shock

A. In rabbits. The granulocytopenic response to irreversible hemorrhagic shock (Table III, B) in rabbits is quite comparable to the response to a lethal dose of endotoxin, except that the absolute lymphocytosis, unlike the granulocytopenia, does not persist until death. The extreme depletion of granulocytes is evident within one minute after inducing shock. In reversible shock return to the normal count begins shortly after transfusion (Table III, A). By the next day there is a leukocytosis, and the granulocyte count is far above normal. The shed blood in the reservoir which is used for the transfusion shows a moderate drop in the total and granulocyte count before it is transfused. This drop is probably accounted for by the fact that the granulocytopenia is present before most of this blood has left the circulation. Even if the transfused leukocytes, which are functionally intact by *in vitro* test (7), remain in the axial stream, they do not increase the count.

TABLE V

Granulocytopenic response during hemorrhagic shock in rabbits pretreated with antibiotics (six experiments)

Time	Mean no. of granulocytes	Approximate S. E. 23%	Readings as % of pretreatment levels
hrs.			
0	4,820	1,109	
1/12	212	49	4.4
1/2	158	36	3.3
1	240	55	5.0
2	1,390	320	28.8
3	2,930	624	60.8
4	3,240	745	67.2

Comment. Rabbits tolerant to endotoxin are also tolerant to hemorrhagic shock, and when exposed to hemorrhagic shock their plasma does not develop a factor toxic to a vulnerable recipient (7). If endotoxins are the chief cause of granulocytopenia in hemorrhagic shock, one would expect the granulocytes in tolerant rabbits in shock to behave as they do when given endotoxin (*cf.* Experiment 2). Table III, C shows that this expectation was confirmed, *i.e.*, the granulocytopenia is relatively less severe in animals tolerant to hemorrhagic shock than in those not tolerant. By the end of the second hour return toward normal is already in evidence, and is near normal when shock is terminated by transfusion at six hours (7).

TABLE VI

Leukopenic response of dogs and rabbits to single I.V. dose of epinephrine *

Time	Mean granulocyte count	Approx. S. E. 29% (18%)	Readings as % of pretreatment level	Mean total white cell count	Approx. S. E. 12% (13%)	Readings as % of pretreatment level
hrs.						
0	4,677 (12,676)	1,356 (2,282)		7,925 (17,220)	951 (2,239)	
1/6	2,952 (10,900)	856 (1,962)	63 (80)	8,184 (14,723)	982 (1,914)	103 (86)
1/2	1,932 (6,545)	560 (1,178)	41 (52)	6,855 (10,422)	823 (1,355)	86 (61)
1	1,449 (5,755)	420 (1,036)	31 (36)	6,775 (9,954)	813 (1,294)	86 (58)
2	1,991 (7,535)	577 (1,356)	43 (59)	5,901 (11,272)	708 (1,465)	74 (65)
4	4,865 (13,020)	1,411 (2,344)	103 (103)	7,889 (17,200)	947 (2,236)	99 (99)

* Figures in parentheses are for nine dogs; figures not in parentheses are for seven rabbits.

B. In dogs. The general trend is the same in dogs as in rabbits, but it is far less marked in the dog (Tables III, A and B).

Comment. If leukopenia is caused by endotoxin in the dog as well as in the rabbit, the lesser response in the dog may reflect a more efficient bone marrow response, a less vulnerable granulocyte, or a lesser amount of circulating endotoxin. The latter seems unlikely if the gross pathology of the shock (especially the hemorrhagic necrosis of the intestinal mucosa) is an index of the severity of the endotoxemia.

Experiment 4—Effect of toxic plasma from irreversibly shocked rabbits injected into normal recipients

Normal plasma did not produce a fall in the total or granulocyte count.

The response to a single dose of toxic plasma is like that to a small dose of endotoxin (Table IV). In no instance did the low granulocyte count last longer than four hours.

Daily doses of toxic plasma given for seven successive days produced the same initial fall in the granulocyte count, but by the fifth day, as with repeated daily doses of endotoxin, the count returned to normal more rapidly than in non-tolerant rabbits. The more rapid return to normal in rabbits tolerant to toxic plasma suggests a more rapid removal of endotoxin from injected plasma.

Experiment 5—Effect of hemorrhagic shock pretreated with nonabsorbable antibiotics

The initial fall in the total and granulocyte count is no less severe in these than in untreated rabbits (*cf.* Table III), but the counts begin a return to normal in two hours. Since the initial fall is in excess of what would appear to be caused by epinephrine alone (see below), one suspects that the blood is not free of endotoxin in these protected animals. From evidence to be published elsewhere there is reason to believe that shortly after giving antibiotics there is a rapid rise in the amount of endotoxin entering the circulation, and, therefore, that the protective effect of antibiotics given daily for several days may be owing to induced tolerance rather than to the prevention of endotoxemia. If this is the case, the severe granulocytopenia in these experiments may be analogous to the granulocyte response in endotoxin-tolerant rabbits as shown in Table II.

Experiment 6—Effect of epinephrine and of norepinephrine

A. In the rabbit (Table VI). The dose employed (0.1 ml. of 1:10,000), or a larger dose, produces a granulocytopenia that is less severe and more transient than that resulting from a very small dose of endotoxin. Thus one may conclude that the granulocytopenic curve in hemorrhagic shock reflects the action of epinephrine as well as endotoxin.

B. In the dog. In the dog the dose employed (0.8 ml. 1:1000) produced no change until after the transient period of hypertension, when the response was quite like that in the rabbit; but recovery was a little more rapid than in the rabbit (Table VI).

Norepinephrine had no effect on the granulocyte response.

C. Effect of pretreatment with dibenamine (rabbit). This drug given in a dose of 20 mg. per Kg. produces maximal adrenergic blockade within 25 minutes. In the normal animal it produces no immediate effect on the count, but a leukocytosis appears after 24 hours.

Epinephrine given three hours after dibenamine failed to evoke a granulocytopenia. But endotoxin in both sublethal (0.0004 MLD per 100) and lethal (1.2 MLD per 100) doses produced the same changes as in the non-dibenaminized rabbit. Whereas two of five rabbits receiving the lethal dose survived, there were no survivors among the non-dibenaminized rabbits receiving this dose. The two survivors showed a marked leukocytosis after 24 hours.

In hemorrhagic shock dibenamine did not prevent a profound and persistent granulocytopenia in four rabbits, but three showed no granulocytopenia and two showed a very transient granulocytopenia (30 minutes).

DISCUSSION

The flight of granulocytes from the circulating blood of the rabbit in response to the intravenous injection of endotoxin has been previously described by others, notably Delauney and associates (5) and Bennett and Beeson (8). Delauney

stated that the onset of the leukopenia occurred one hour after intravenous injection. Berthrong and Cluff (9) observed that the granulocytopenia following sublethal doses of endotoxin was maximal within one to two hours, and was replaced by a polymorphonuclear leukocytosis at six to seven hours. Following sublethal or lethal doses of endotoxin we have observed that the granulocytopenia is maximal or nearly so within a few minutes.

The striking parallelism between the changes in the total and differential counts in experimental endotoxemia and in hemorrhagic shock, both in time of onset and degree and duration, suggests a common mechanism in both. The immediate granulocytopenic response to endotoxin signifies a direct effect of endotoxin on the granulocytes, or on the vessels to which they adhere. Since this occurs even in the dibenaminized animal it cannot be, as Delauney (5) believes, a wholly epinephrine-dependent phenomenon.

If this is so, and the phenomena involved are the same in hemorrhagic shock, one must conclude that an endotoxemia is present within minutes after induction of hemorrhagic shock. That this is the case is also suggested by recent experiments to be reported showing that in thorotrast-pretreated rabbits hemorrhagic shock of only *five minutes' duration* elicits a severe Shwartzman reaction and causes death. If the immediate and severe granulocytopenic response in hemorrhagic shock, like the granulocytopenia of endotoxemia, cannot be explained as being wholly or primarily due to epinephrine, the prevention of the granulocytopenic response in five of nine dibenaminized animals in hemorrhagic shock would seem to be owing to some property of dibenamine not related to adrenergic blockade.

One cannot exclude the possibility that a leukopenic agent other than endotoxin or epinephrine may be operating in hemorrhagic shock. The fact that the leukopenia occurs so rapidly after the onset of shock would appear to exclude any leukopenic agent which develops only in consequence of tissue injury caused by prolonged duration of the shock state. Until evidence for some other type of leukopenic agent is forthcoming, it appears that the flight of granulocytes in hemorrhagic shock can be considered to be mainly a manifestation of endotoxemia. According to Braude and co-workers (10, 11) endotoxin attaches to the granulocytes, which then move to the liver and lungs, and so acts to transport endotoxin to the R.E. system. We have observed the deposition of the leukocytes in the capillaries of the liver, lung and spleen almost immediately after inducing shock or injecting toxin. Many of them can be seen *in situ* 24 hours later in process of disintegration or phagocytosis by macrophages. These cells are functionally damaged because they do not move to a site of challenge (*e.g.*, to bacteria injected into the lung or liver just prior to the flight of the granulocytes[2]). From *in vitro* observations we know that endotoxin or shock plasma produces a severe though reversible morphologic injury, which may make the cell more readily available for ingestion by the R.E. cells; but in addition, the granulocytes exhibit a severe depression of their phagocytic and bacteriostatic capacity (6). These observations indicate that the flight of these cells is not a protective phenomenon, unless perchance the flight is an essential means for delivery of endotoxins to the R.E. system. The deposition of the granulocytes in the R.E. system, though probably achieved by an increase in the "stickiness" of the capillary walls, resembles the process of agglutination described by Wood and Smith in the response of circulating granulocytes to a local inflammatory stimulus (12). But in the latter case the adherent cell manages to penetrate the capillary wall, reach the site of challenge, and continue to function. Meanwhile there is no dearth of circulating cells to serve the defense needs of other areas. In shock, on the other hand, all other areas are less well defended by virtue of the low circulating count, and the inability of the bone marrow to supply the deficit. In human shock leukopenia or leukocytosis has been observed (13). Presumably this depends on the state of bone marrow activity preceding the onset of the shock state, and the sensitivity of this system to depression by circulating toxins. In the rabbit this sensitivity is high, in the dog it is less, and in man the observations are too few to provide a definite picture.

SUMMARY AND CONCLUSIONS

The circulating granulocytes respond to the intravenous injection of endotoxin or to hemor-

[2] Unpublished observations.

rhagic shock by an almost immediate flight into the capillaries of the lung, liver and spleen, where they appear to remain and eventually undergo autolysis or phagocytosis by macrophages. The epinephrine response to shock or to endotoxin also produces a flight of the granulocytes. But because the flight due to epinephrine alone is much less severe and more transient, it cannot wholly account for the phenomenon. This is further demonstrated by the fact that dibenamine does not block the flight induced by endotoxins. But because dibenamine frequently minimizes or prevents the extreme granulocytopenic response to hemorrhagic shock, its protective effect may lie not only in its anti-epinephrine effect, but also in the preservation of the detoxifying potential of the tissues by virtue of the better blood flow which this drug effects in hemorrhagic shock. The altered granulocytopenic response to endotoxin in animals tolerant to endotoxin and to hemorrhagic shock is attributable to the very rapid detoxification of endotoxin in such animals.

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METABOLIC STUDIES WITH A NEW SERIES OF 1,4-DIENE STEROIDS. I. EFFECTS IN ADDISONIAN SUBJECTS OF PREDNISONE, PREDNISOLONE, AND THE 1,2-DEHYDRO ANALOGUES OF CORTICOSTERONE, DESOXYCORTICOSTERONE, 17-HYDROXY-11-DESOXYCORTICOSTERONE, AND 9α-FLUOROCORTISOL *

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Prednisone and prednisolone,[1] the first steroids in this series (2), were shown to possess anti-inflammatory activities much greater than those of the parent steroids, cortisone and cortisol (3). The present study was undertaken to delineate further the change in physiologic function resulting from the introduction of the extra double bond. A number of later reports of the metabolic effects of three of these steroids are now available (4–6).

METHODS

Balance studies were carried out on three female patients with Addison's disease. In all patients the diagnosis had been established clinically, and confirmed by the findings of control 17-ketosteroids and 17-hydroxycorticoid excretion of less than 1 mg. per day, not rising with adrenocorticotropic hormone (ACTH). Each had been maintained in good clinical condition with oral cortisone and daily intramuscular desoxycorticosterone injections until eight to 10 days before the first steroid was given in these studies. No further supportive treatment was given. The diet and fluid intake were kept constant throughout each study. A liquid diet was used throughout the study on L. M. B. and in one study on A. M. B. Stools were collected in four or six day periods and urine in one day pools. Stools, diets and urine were analyzed for nitrogen (7), phosphorus (8), calcium (9), potassium (10) and sodium (10). Blood was drawn at appropriate intervals for the determination of circulating eosinophiles and serum sodium, potassium, carbon dioxide, calcium and phosphorus. Studies of the acute effects of steroids on circulating eosinophiles over four hour periods were also done in one Addisonian and one normal subject. Four

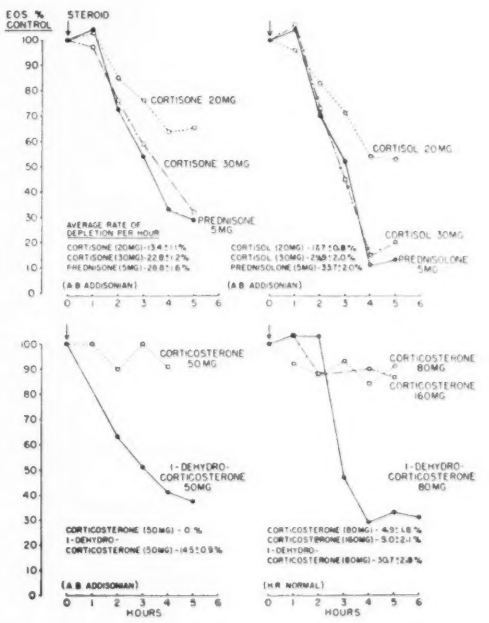

FIG. 1. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON CIRCULATING EOSINOPHILES FOUR HOURS AFTER INJECTION

Steroids were given intravenously over 10 minute periods. The average rate of depletion per hour has been determined from the slope of the linear regression (11).

* Part of this material has been presented previously (1). The names "metacortandracin" and "metacortandralone" have since been replaced by "prednisone" and "prednisolone."

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[1] The trivial names, together with the chemical names, of the steroids studied are as follows: prednisone, 17α,21-dihydroxypregna-1,4-diene-3,11,20-trione; prednisolone, 11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; cortisone, 17α,21-dihydroxypregn-4-ene-3,11,20-trione; cortisol, 11β,17α,21-trihydroxypregn-4-ene-3,20-dione; desoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; 9α-fluoroprednisolone, 9α-fluoro,11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione.

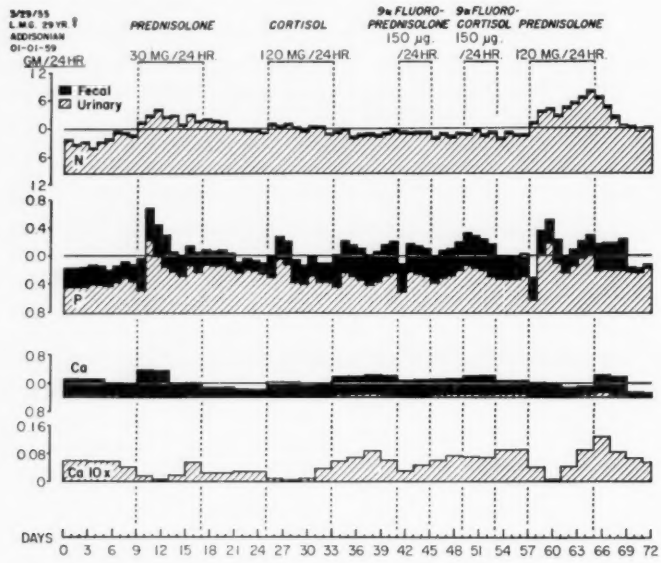

FIG. 2. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON NITROGEN, PHOSPHORUS AND CALCIUM BALANCES IN L. M. G.

Balance data in this and subsequent charts are plotted as follows: Intake is plotted downwards from the 0 line, urinary values are plotted upwards from the intake line, and fecal values are superimposed on them. Thus, positive balance is indicated by a clear area below the 0 line and negative balance by a shaded area above it.

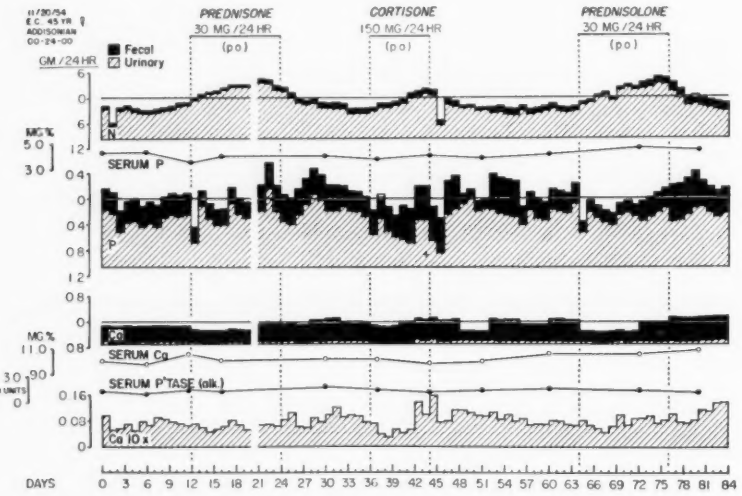

FIG. 3. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON NITROGEN, PHOSPHORUS AND CALCIUM BALANCES, SERUM PHOSPHORUS, CALCIUM AND ALKALINE PHOSPHATASE IN E. C.

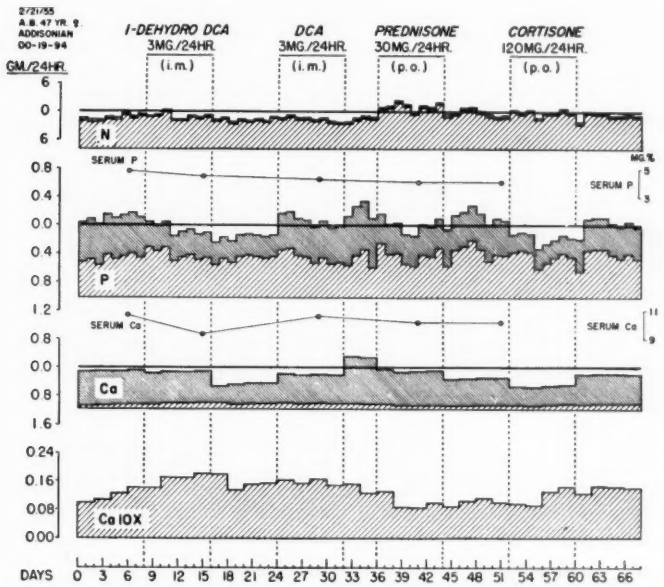

FIG. 4. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON NITROGEN, PHOSPHORUS AND CALCIUM BALANCES, SERUM PHOSPHORUS AND CALCIUM IN A. M. B.

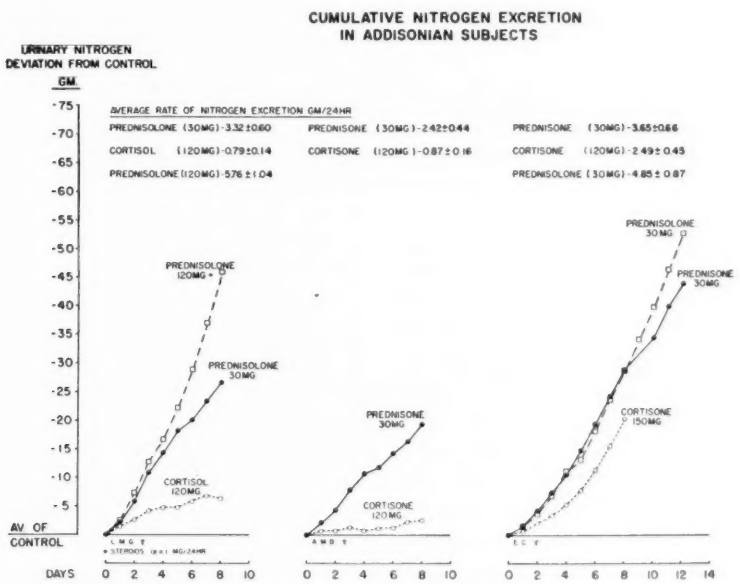

FIG. 5. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON NITROGEN EXCRETION EXPRESSED AS THE MEAN DAILY DIFFERENCE BETWEEN TREATMENT AND CONTROL PERIODS

The daily deviation from control is plotted cumulatively. The daily average rate of nitrogen excretion has been determined from the slope of the linear regression (11).

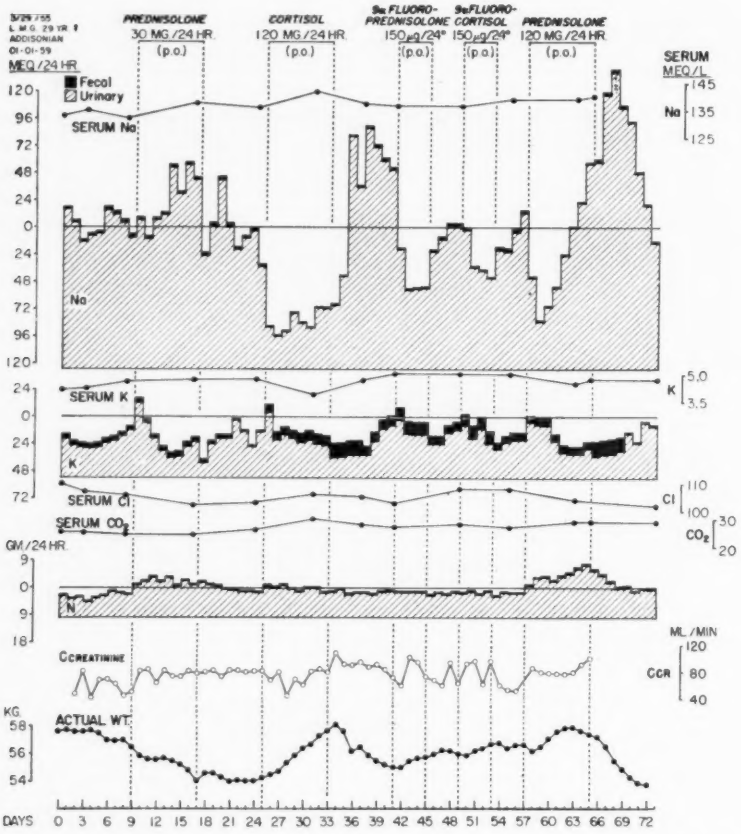

FIG. 6. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON SODIUM, POTASSIUM AND NITROGEN BALANCES, SERUM SODIUM, POTASSIUM, CHLORIDE, CARBON DIOXIDE, CREATININE CLEARANCE AND BODY WEIGHT IN L. M. G.

separate pipettes were filled with blood at the control and each of five hours following the intravenous administration of the steroid and counted separately. Inulin and para-aminohippurate clearances were used as measures of glomerular filtration rate and renal plasma flow, respectively, in one subject. In the other two, 24 hour clearances of endogenous creatinine were used as an index of glomerular filtration rate. Steroids were given orally, one-quarter of the daily dose every six hours.

RESULTS

The results are shown graphically in Figures 1 through 13.

Inasmuch as anti-inflammatory activity is found in association with eosinopenic and anti-anabolic or catabolic activity, the effects of these steroids on eosinophiles and on the constituents of protoplasm were examined.

1. The eosinopenic effect

Figure 1 shows the effects of single doses of steroids administered intravenously on circulating eosinophiles. The 1,4-diene steroids were four to six times as active as the parent compounds.[2] This was so for 1-dehydrocorticosterone

[2] It was on the basis of these studies that the factor "4" was originally chosen for the comparative study of anti-inflammatory activities of prednisone, prednisolone and their parent compounds in rheumatoid arthritis. In fact (*vide infra*), a higher factor is probably more correct.

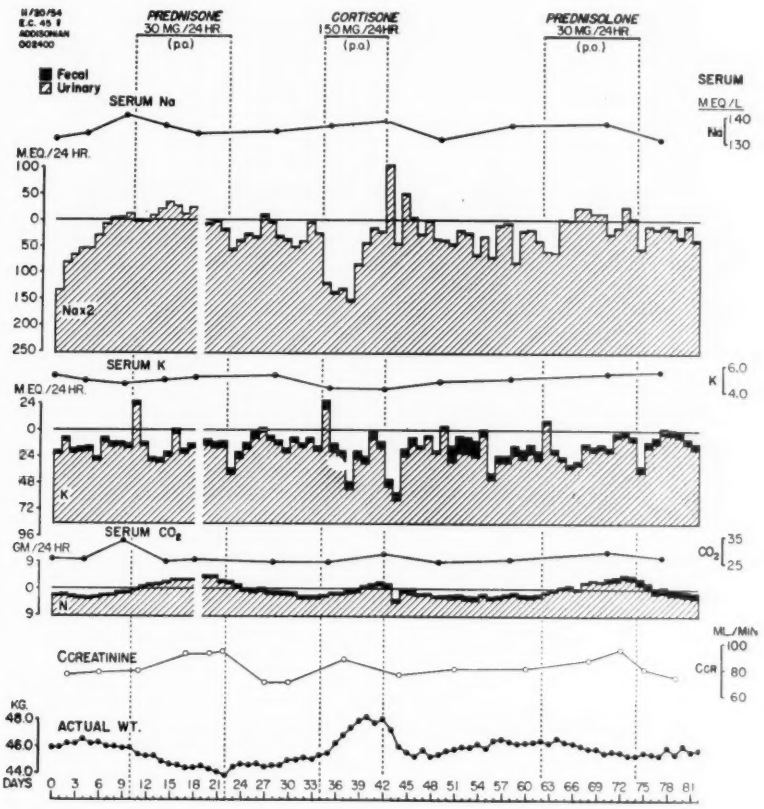

FIG. 7. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON SODIUM, POTASSIUM AND NITROGEN BALANCES, SERUM SODIUM, POTASSIUM, CARBON DIOXIDE, CREATININE CLEARANCE AND BODY WEIGHT IN E. C.

which lacks a hydroxyl group at carbon 17 as well as for prednisone and prednisolone which possess it.

II. Nitrogen metabolism

Prednisone and prednisolone, in doses of 30 mg. a day, caused an increase in urinary nitrogen excretion and a negative nitrogen balance (Figures 2 through 4). The loss of nitrogen with 30 mg. of the 1,4-diene steroids was considerably greater than that with the parent compounds, cortisone and cortisol, at doses four or five times as large. These results are summarized in Figure 5. As judged from the effects on nitrogen metabolism, prednisone and prednisolone, administered orally, appeared to be 10 to 17 times [parallel line assay method (11)] as active as the parent steroids.

III. Phosphorus metabolism

Prednisone and prednisolone, in doses of 30 mg. a day, had little effect on phosphorus metabolism. In all cases but one there was a transient fall in urinary phosphorus on the first day of therapy and a transient rise on the second day. In the remaining case (Figure 4) urinary phosphorus rose on the first day. In all cases but one urinary phosphorus fell after therapy was stopped. In no case was there a loss of phosphorus commensurate [N : P = 0.068 Gm. per Gm. (12)] with the loss of nitrogen. The effects of the 1,4-diene steroids on phosphorus were quite comparable with those

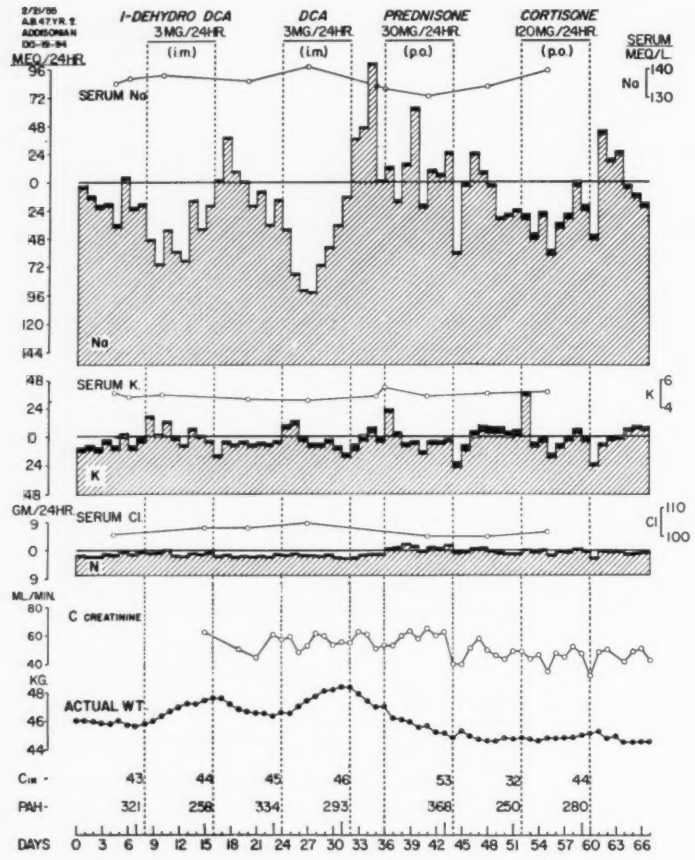

FIG. 8. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON SODIUM, POTASSIUM, AND NITROGEN BALANCES, SERUM SODIUM, POTASSIUM, CHLORIDE, BODY WEIGHT, CREATININE CLEARANCE, INULIN CLEARANCE AND PARA-AMINOHIPPURATE CLEARANCE IN A. M. B.

of the parent compounds at doses four or five times as great. Wide fluctuations in fecal phosphorus, apparently not related to steroid thereapy, induced corresponding fluctuations in phosphorus balance and made it difficult to interpret. A relative retention of phosphorus during steroid-induced nitrogen loss has been attributed to concomitant glycogen formation (13). On the other hand, the phenomenon may represent the loss of relatively phosphorus-poor protein.

IV. Calcium metabolism

With prednisone and prednisolone, in doses of 30 mg. a day, there was a transient fall of urinary calcium in two cases (Figures 2 and 4) and no change in the other (Figure 3). Fecal calcium was lower than that in the control periods during three of the four periods studied at this dose. With cortisone and cortisol, in doses four to five times as large, there was a fall of urinary calcium. This was accompanied by sodium retention and weight gain. There was a similar fall of urinary calicum, with sodium retention, with prednisolone at a dose of 120 mg. a day (Figure 2). The calcium retention with cortisone, cortisol and with the 120 mg. dosage of prednisolone did not persist throughout treatment, the "escape" therefrom closely paralleling that of sodium (*vide infra*).

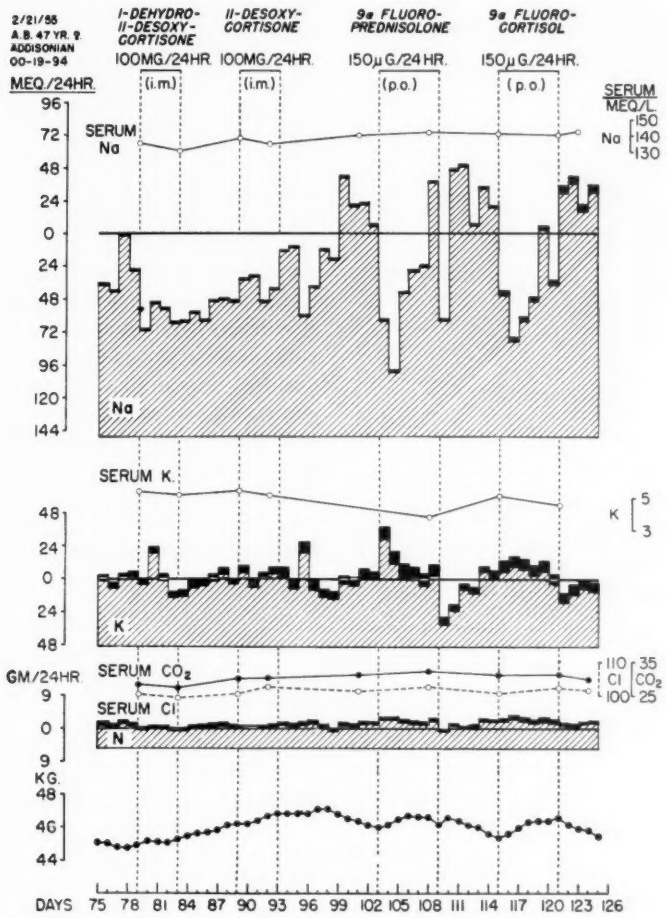

FIG. 9. THE EFFECT OF 1-DEHYDRO-11-DESOXYCORTISONE, 9α-FLUOROPREDNISOLONE AND THE PARENT COMPOUNDS ON SODIUM, POTASSIUM AND NITROGEN BALANCES, SERUM SODIUM, POTASSIUM, CARBON DIOXIDE, CHLORIDE AND BODY WEIGHT IN A. M. B.

The amounts of calcium gained and lost were approximately those normally found in the volumes of extracellular fluid (ECF) gained and lost concomitantly (Ca:Na = 0.36 mg. per mEq. in ECF).

V. Potassium metabolism

Prednisone and prednisolone produced a transient increase of urinary potassium, with negative balance, on the first day of therapy: With 120 mg. of prednisolone a day this effect persisted for three days. When treatment was stopped, there was a transient decrease of urinary potassium, with positive balance, for one or two days. The same changes occurred with cortisone and cortisol at four to five times the dose. The results are summarized in Figure 12. These changes in potassium balance were not accompanied by commensurate [N:K = 2.7 mEq. per Gm. (12)] changes in nitrogen balance and in most instances precede by a day corresponding changes in urinary phosphorus.

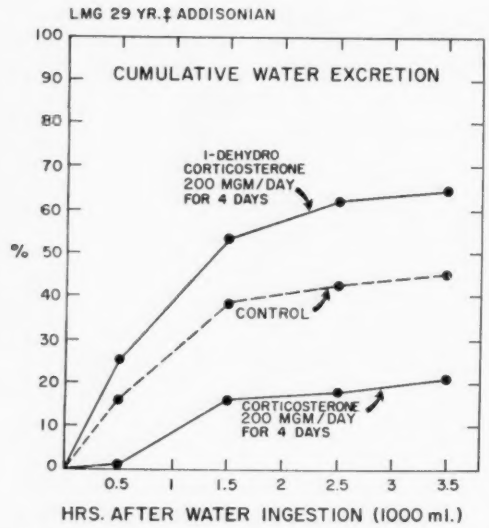
LMG 29 YR.‡ ADDISONIAN
CUMULATIVE WATER EXCRETION
1-DEHYDRO CORTICOSTERONE 200 MGM/DAY FOR 4 DAYS
CONTROL
CORTICOSTERONE 200 MGM/DAY FOR 4 DAYS
%
HRS. AFTER WATER INGESTION (1000 ml.)

FIG. 10. THE EFFECT OF 1-DEHYDROCORTICOSTERONE AND THE PARENT COMPOUND ON CUMULATIVE WATER EXCRETION FOLLOWING A STANDARD WATER LOAD IN L. M. G.

VI. Sodium metabolism

With prednisone and prednisolone, in doses of 30 mg. a day, there was a slight (Figures 7 and 8) to moderate (Figure 6) rise in urinary sodium, with negative sodium balance. With cortisone and cortisol, at doses four to five times as large, there was slight (Figure 8) to marked (Figures 6 and 7) sodium retention. In the experiment with cortisone (Figure 7) there was "escape" from sodium retention during therapy. With prednisolone, at a dose of 120 mg. a day (Figure 6), there was early sodium retention followed by "escape" such that there was substantial sodium loss during the last two days of therapy. Similar results were obtained by other workers (4–6).

With 1-dehydrodesoxycorticosterone acetate (1-dehydro-DCA), at a dose of 3 mg. a day (Figure 8), there was moderate sodium retention. With desoxycorticosterone acetate (DCA), at the same dosage, there was the same degree of sodium retention.

With 9α-fluoroprednisolone, at a dosage of 150 μg. a day (Figures 6 and 9), there was moderate sodium retention in two subjects: With 9α-fluorocortisol, at the same dose, there was the same degree of sodium retention.

VII. Glomerular filtration rate (GFR)

With prednisone and prednisolone, in doses of 30 mg. a day, the inulin clearance rose in A. B. (Figure 8), and the endogenous creatinine clearance rose in E. C. (Figure 7) and in L. M. B. (Figure 6). With cortisone and cortisol, in doses four or five times as large, the corresponding inulin and creatinine clearances rose in A. B. and in E. C., but not in L. M. G. The fluctuations in 24 hour creatinine clearances were such as to render the changes of questionable statistical significance; the observations on inulin clearance with prednisone and cortisone were limited in number. Figure 13 shows results of therapy with prednisone and prednisolone, 60 to 70 mg. a day, on inulin clearance in four other subjects with normal renal and adrenal cortical function: For two of them the effect of cortisone and cortisol, at doses four times as large, is also shown. With both types of treatment comparable rises were produced.

DISCUSSION

The results indicate that the introduction of a double bond at position 1–2 increases the eosinopenic and anti-anabolic or catabolic activity of steroids originally possessing these properties. These properties are always associated with oxygen functions at carbon 11 and carbon 17. When these are not present, eosinopenic and anti-anabolic or catabolic properties are absent.

With the 1–2 double bond in the absence of an oxygen function at carbon 11 (1-dehydro-11-desoxycortisol, see Figure 9), no anti-anabolic or

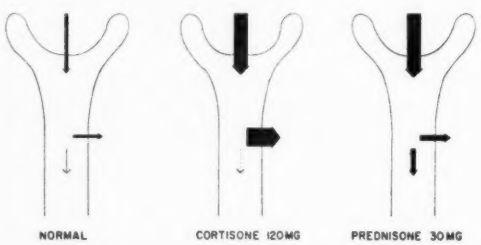

FIG. 11. SCHEMA TO ILLUSTRATE THE DIFFERENT EFFECTS OF A 1,4-DIENE STEROID (PREDNISONE) AND THE PARENT COMPOUND (CORTISONE) ON RENAL FUNCTION

The arrows represent sodium, the upper one representing filtered sodium, the middle one reabsorbed sodium and the lower one urinary sodium.

catabolic activity was found. With the 1–2 double bond in the absence of an oxygen function at carbon 17 (1-dehydrocorticosterone, see Figure 1), eosinopenic activity appeared in a steroid whose parent compound lacked this property. The data in Figure 10 suggest that corticosterone also acquired the ability to promote a water diuresis with the introduction of a 1–2 double bond; this property is associated with the anti-anabolic or catabolic activity of steroids. It thus appears that enhancement of anti-anabolic or catabolic (and anti-inflammatory) activity by 1,2-dehydrogenation depends upon the presence of an oxygen atom at carbon 11.

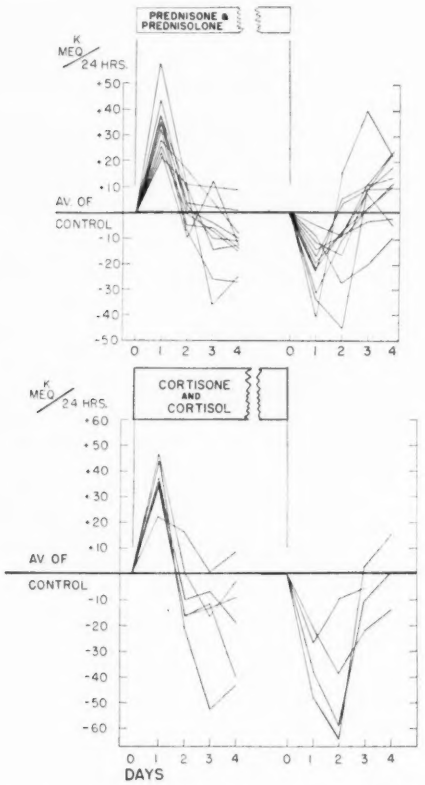

FIG. 12. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON URINARY POTASSIUM, EXPRESSED AS A DEVIATION FROM THE AVERAGE OF THAT OF THE CONTROL PERIODS OF THE FIRST FOUR DAYS ON AND THE FIRST FOUR DAYS AFTER TREATMENT

The data include all the studies reported by the present paper and studies in normal subjects.

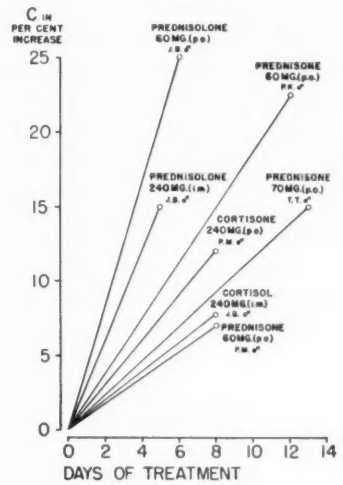

FIG. 13. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON INULIN CLEARANCE IN FOUR SUBJECTS WITH NORMAL RENAL AND ADRENAL CORTICAL FUNCTION

The property of anti-anabolic or catabolic steroids to increase the glomerular filtration rate appeared to be enhanced in the 1,2-dehydrogenated steroids in parallel with the enhancement of anti-anabolic or catabolic activity itself.

Whereas 1,2-dehydrogenation thus affects the properties of 11,17-oxygenated steroids quantitatively by increasing anti-anabolic or catabolic activity, it appears to affect them qualitatively as regards sodium metabolism. Whereas sodium retention generally occurs with the parent compounds, cortisone and cortisol, sodium loss is regularly seen with the 1,4-diene derivatives, prednisone and prednisolone.

To explain the difference in the effects on sodium metabolism it is not necessary to postulate that unsaturation at carbon 1–2 affects tubular sodium transport directly: In the comparison of DCA with 1-dehydro-DCA no such effect was apparent. The sodium loss produced with the 1, 4-dienes is more likely a result of the increase in glomerular filtration rate, and thus of tubular sodium "load," which these steroids produce. This effect is correlated with anti-anabolic or catabolic activity of steroids (14). Since 1,2-dehydrogenation enhances this type of activity, it could confer on a steroid the ability to cause

sodium diuresis without having any effect on its ability to potentiate tubular sodium transport. Figure 11 represents this concept diagrammatically. If prednisone, at a dosage of 30 mg., can increase glomerular filtration rate as much as cortisone, at a dosage of 120 mg. (as our data suggest), it can produce sodium loss if the effect on tubular transport, milligram for milligram, is the same in the two steroids. When 9α-fluoroprednisolone was compared with 9α-fluorocortisol, in doses too small to affect glomerular filtration rate (Figures 6 and 9), the effects on sodium balance were the same.

SUMMARY AND CONCLUSIONS

The effect of unsaturation by 1,2-dehydrogenation on the activity of 21-carbon steroids was explored with balance and renal studies in three patients with Addison's disease. Unsaturation at carbon 1–2 resulted in an increase in anti-anabolic or catabolic properties in those steroids with oxygen at carbons 11 and 17 (for example, cortisone and cortisol) and in the appearance of such properties in a steroid with oxygen at carbon 11 but not at carbon 17 (corticosterone).

Unsaturation at carbon 1–2 resulted in a loss of sodium-retaining properties in those steroids in which it enhanced anti-anabolic or catabolic properties, when given in doses large enough to increase the glomerular filtration rate; this loss of sodium-retaining properties is ascribed to the "glomerulotubular" imbalance so induced.

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METABOLIC STUDIES WITH A NEW SERIES OF 1,4-DIENE STEROIDS. II. EFFECTS IN NORMAL SUBJECTS OF PREDNISONE, PREDNISOLONE, AND 9α-FLUOROPREDNISOLONE *

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(Submitted for publication May 13, 1958; accepted December 4, 1958)

1,2-Dehydrogenation of 21-carbon steroids possessing an oxygen function at carbons 11, 17 and 21 (cortisone and cortisol) results in an enhancement of anti-anabolic or catabolic and anti-inflammatory properties in Addisonian subjects (2). The present study was undertaken to extend these observations to normal subjects and to delineate further the effects of these steroids, in very large doses, on the constituents of protoplasm.

* Part of this material has been presented previously (1). The names "metacortandracin" and "metacortandralone" have since been replaced by "prednisone" and "prednisolone."

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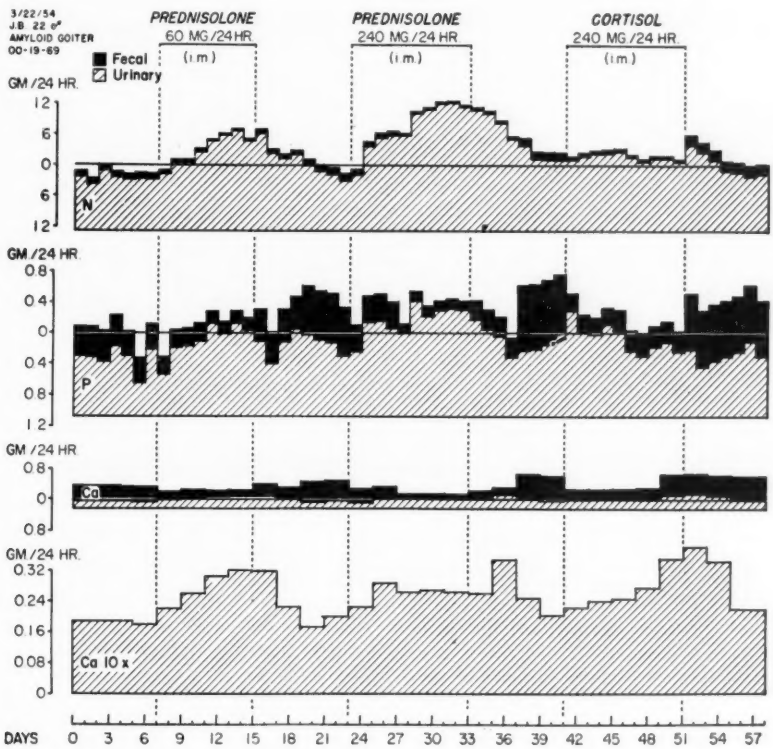

FIG. 1. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON NITROGEN, PHOSPHORUS AND CALCIUM BALANCES IN J. B.

Balance data in this and subsequent charts are plotted as follows: Intake is plotted downwards from the 0 line, urinary values are plotted upwards from the intake line, and fecal values are superimposed on them. Thus, positive balance is indicated by a clear area below the 0 line, and negative balance by a shaded area above it.

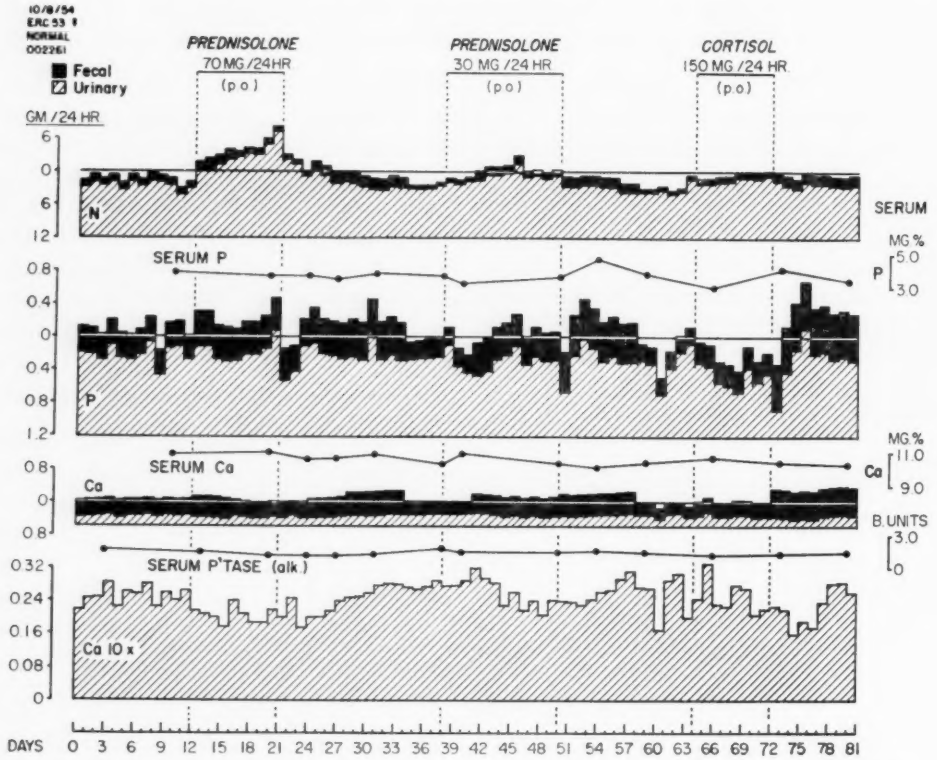

FIG. 2. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON NITROGEN, PHOSPHORUS AND CALCIUM BALANCES, SERUM PHOSPHORUS, CALCIUM AND ALKALINE PHOSPHATASE IN E. C.

METHOD

Balance studies were carried out on three normal subjects. The diet and fluid intake were kept constant. Stools were collected in six or eight day periods and urine in one day pools. Stools, diets and urine were analyzed for nitrogen (3), phosphorus (4), calcium (5), potassium and sodium (6). Urine was also analyzed for 17,21-dihydroxycorticoids (phenylhydrazine-sulfuric acid chromogens) (7) and 17-ketosteroids (8). Blood was drawn at appropriate intervals for the determination of circulating eosinophiles, serum sodium, potassium, carbon dioxide, calcium and phosphorus. Steroids were given orally or intramuscularly, one-quarter of the dose every six hours.

RESULTS

The results are shown graphically in Figures 1 through 11.

During treatment with both the 1,4-diene steroids and the parent compounds the fecal excretion of nitrogen, phosphorus, calcium and potassium was generally less than that in the preceding control periods; after treatment it was generally much greater. The patients complained of constipation during treatment. The large fluctuations in apparent calcium and phosphorus balances resulting from this phenomenon (see, for example, Figures 1 and 2) render balance data relatively less reliable than urinary data for these elements.

I. Nitrogen metabolism

Prednisolone, in doses of 30 mg. a day, caused an increase in urinary nitrogen excretion and a slight negative nitrogen balance (Figure 2). The loss of nitrogen at this dosage was only slightly greater than that with the parent steroid, cortisol, at five times the dose. Prednisone and prednisolone, in doses of 70 mg. a day administered orally, caused a marked increase in urinary nitrogen ex-

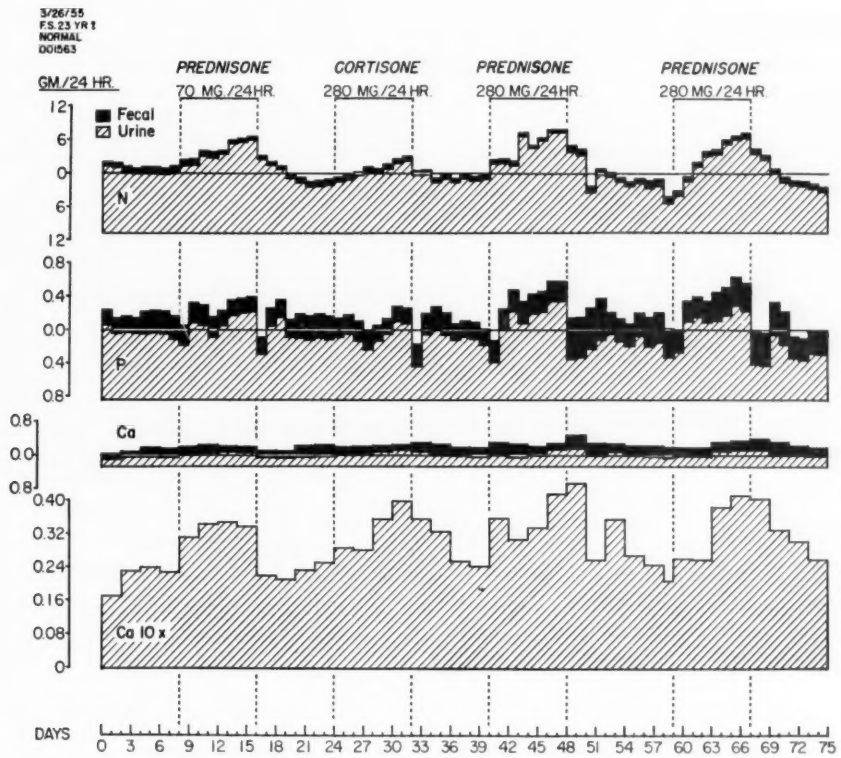

FIG. 3. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON NITROGEN, PHOSPHORUS AND CALCIUM BALANCES IN F. S.

cretion and a negative nitrogen balance (Figures 2 and 3). The loss of nitrogen induced by 70 mg. of the 1,4-diene steroids was considerably greater than that with the parent steroids, cortisol and cortisone, at doses two to five times as large. Prednisone, in doses of 280 mg. a day, caused a large increase in urinary nitrogen and a negative nitrogen balance (Figure 3). The loss of nitrogen at this dosage was greater than that with cortisone at the same dosage. The loss of nitrogen, with 60 and 240 mg. of prednisolone administered intramuscularly (Figure 1), was similar in magnitude to that caused by 70 and 280 mg. of the 1,4-diene steroids administered orally (Figures 2 and 3), but persisted for longer periods following the termination of therapy. The effects of the 1,4-diene steroids and the parent compounds on nitrogen excretion are summarized in Figure 4. As judged from the effects on nitrogen balance, prednisone and prednisolone, administered orally, appear from these studies to be seven to eight times [parallel line assay method (9)] as active as the parent steroids. The effects on nitrogen metabolism of both types of steroids are quantitatively more pronounced in the Addisonian than in the normal subjects (Figure 4). 9α-Fluoroprednisolone and the parent compound, 9α-fluorocortisol, in doses of 3 mg. a day, did not affect nitrogen metabolism (Figure 5).

II. Phosphorus metabolism

Prednisolone, at a dose of 30 mg. a day, had little effect on phosphorus metabolism (Figure 2). The slight apparent negative phosphorus balance seen with this treatment did not differ from that found throughout these studies. Prednisone and prednisolone, in doses of 60 to 280

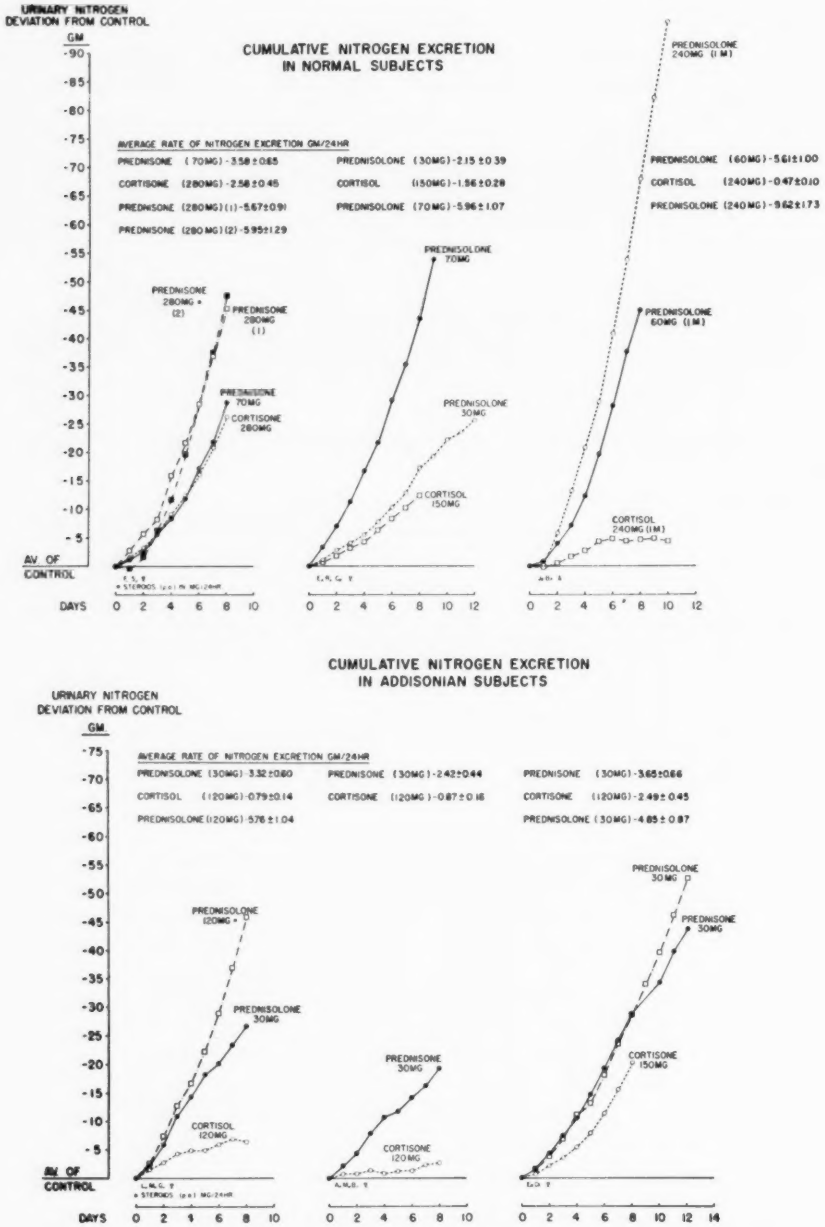

FIG. 4. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON NITROGEN EXCRETION, EXPRESSED AS THE MEAN DAILY DIFFERENCE BETWEEN TREATMENT AND CONTROL PERIODS

The daily deviation from control is plotted cumulatively. Comparable results for three Addisonian subjects are included for comparison. The daily average rate of nitrogen excretion has been determined from the linear regression (9).

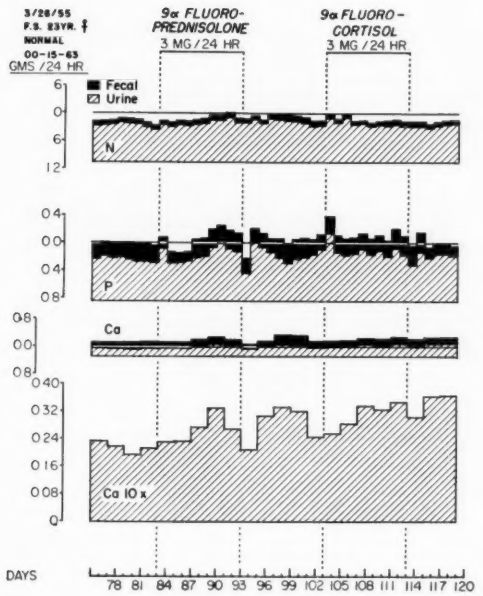

FIG. 5. THE EFFECT OF 9α-FLUOROPREDNISOLONE AND THE PARENT COMPOUND ON NITROGEN, PHOSPHORUS AND CALCIUM BALANCES IN F. S.

mg. a day, caused an increase of urinary phosphorus which was not apparent until the second day of medication (Figures 1 and 3). Indeed, in three instances urinary phosphorus fell on the first day. In all instances urinary phosphorus fell promptly after treatment was stopped. Cortisone and cortisol, in doses of 150 to 280 mg. a day, caused a decrease in urinary phosphorus in two instances and no change in the third: The increase in negative phosphorus balances seen with the 1,4-diene steroids was not seen with the parent compounds. Even when considerable phosphorus loss was produced by the 1,4-diene steroids, it was disproportionately small compared to the nitrogen loss (10, 11).

III. Calcium metabolism

Prednisolone, at a dose of 30 mg. a day, had little effect on calcium metabolism (Figure 2). The slight apparent negative calcium balance with this medication did not differ from that found throughout these studies. Prednisone and prednisolone, in doses of 60 to 280 mg. a day, caused an increase of urinary calcium in five of the six periods studied (Figures 1 and 3) with return to control values in one day after oral medication and in two to three days after intramuscular medication. In the remaining period, urinary calcium fell during the treatment (Figure 2). With cortisone and cortisol, in doses of 240 and 280 mg. a day, there was an increase in urinary calcium with a subsequent fall on withdrawal of medication (Figures 1 and 3). With cortisol, at a dose of 150 mg. a day, urinary calcium did not change significantly (Figure 2). With 9α-fluoroprednisolone and 9α-fluorocortisol, in doses of 3 mg. a day, there was a rise of urinary calcium (Figure 5).

IV. Potassium metabolism

Prednisone and prednisolone, in doses of 30 to 280 mg. a day, caused transient increases of urinary potassium, with negative balance, on the first day of treatment (Figures 6 through 8). When treatment was discontinued, there was a transient decrease of urinary potassium, with positive balance, for one to three days. The same changes occurred with cortisone and cortisol in doses of 150 to 280 mg. a day (Figures 6 through 8). In no instance were the changes in potassium balance accompanied by commensurate changes in nitrogen balance: They occurred even when there were no reciprocal changes in sodium balance.

9α-Fluoroprednisolone and 9α-cortisol, in doses of 3 mg. a day, caused similar marked transient increases of urinary potassium, with negative balance, on the first day of treatment (Figure 9). On the fourth subsequent day there occurred a second rise in the urinary potassium, with negative balance, which persisted until withdrawal of treatment: The serum potassium fell markedly with both steroids. A prompt decrease of urinary potassium, with positive balance, occurred when treatment was discontinued. These latter periods of negative and positive balance were associated with reciprocal changes in sodium balance (*vide infra*).

V. Sodium metabolism

Prednisone and prednisolone, in doses of 30 to 70 mg. a day, produced increases in urinary sodium with negative sodium balance. In two in-

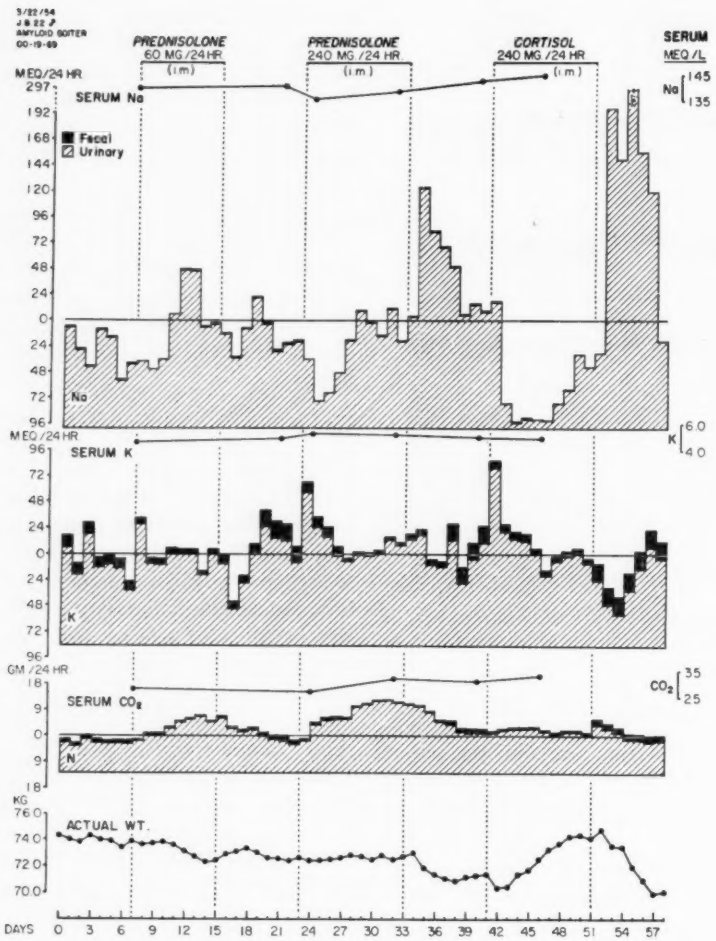

FIG. 6. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON SODIUM, POTASSIUM AND NITROGEN BALANCES, SERUM SODIUM, POTASSIUM, CARBON DIOXIDE AND BODY WEIGHT IN J. B.

stances this did not occur until the fifth day of treatment: In one it was preceded by slight sodium retention (Figures 6 through 8). In doses of 240 to 280 mg. a day, prednisone and prednisolone caused sodium retention which did not persist beyond the fourth day and was, in one instance, followed by significant sodium loss despite continued treatment (Figures 6 and 8). The effect of doubling the sodium intake on the response to 280 mg. of prednisone is shown in Figure 8. On the higher sodium intake the early retention and the late loss of sodium were quantitatively much greater.

Cortisone and cortisol, in doses of 150 and 280 mg. a day, produced quantitatively much greater retention of sodium and allowed relatively little "escape" during therapy (Figures 6 and 8).

9α-Fluoroprednisolone and 9α-fluorocortisol, in doses of 3 mg. a day, caused marked sodium retention (Figure 9), which was accompanied (*vide supra*) by potassium loss and hypokalemia. Quantitatively, the effects of the two steroids

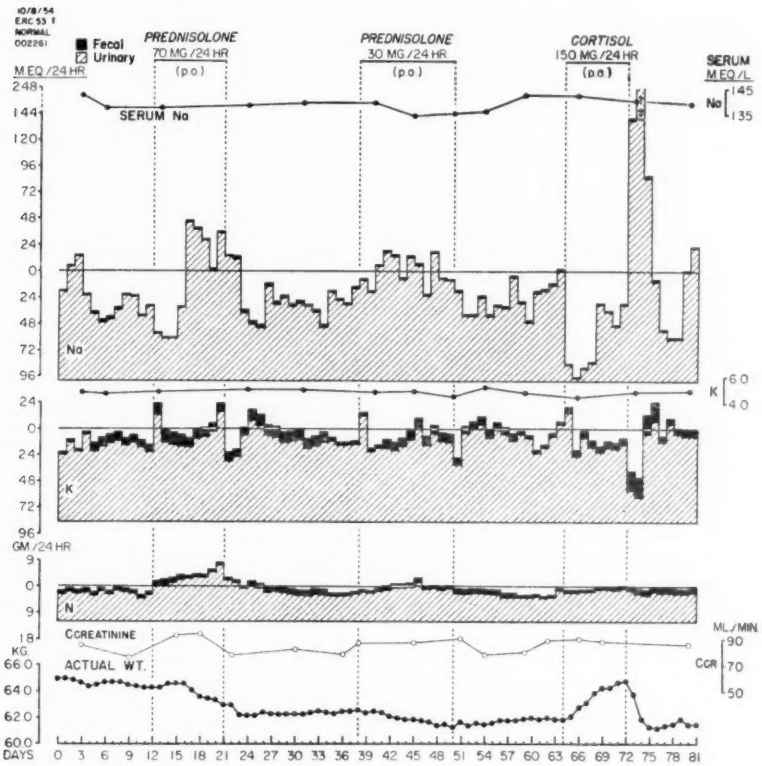

FIG. 7. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON SODIUM, POTASSIUM AND NITROGEN BALANCES, SERUM SODIUM, POTASSIUM, CREATININE CLEARANCE AND BODY WEIGHT IN E. C.

were comparable. In both cases there was partial "escape" from the sodium-retaining effect with continued therapy.

VI. 17-Ketosteroids

In a dose of 30 mg. a day, prednisolone caused a slow decrease in urinary 17-ketosteroid excretion, followed by a prompt return to control values after treatment was stopped (Figure 10). At a dose of 70 mg. a day, it caused a prompt decrease in urinary 17-ketosteroid excretion, followed by a slow return to control values. At a dose of 150 mg. a day, cortisol caused a transient decrease in urinary 17-ketosteroids, followed by an increase despite continued treatment. After treatment was stopped, urinary 17-ketosteroid excretion decreased to values comparable to those observed during treatment with the 1,4-diene steroids.

VII. Theoretical weight curves

"Theoretical" weight curves based on nitrogen, potassium and sodium balances are presented in Figures 8 and 9. They show excellent correlation with the actual weight curves indicating that there were no unexplained shifts of fluid during these studies.

DISCUSSION

The loss of nitrogen produced by the 1,4-diene steroids and the parent compounds is shown in summary in Figure 4. It is clear that the potency of prednisone and prednisolone, judged from their effects upon nitrogen metabolism, is considerably

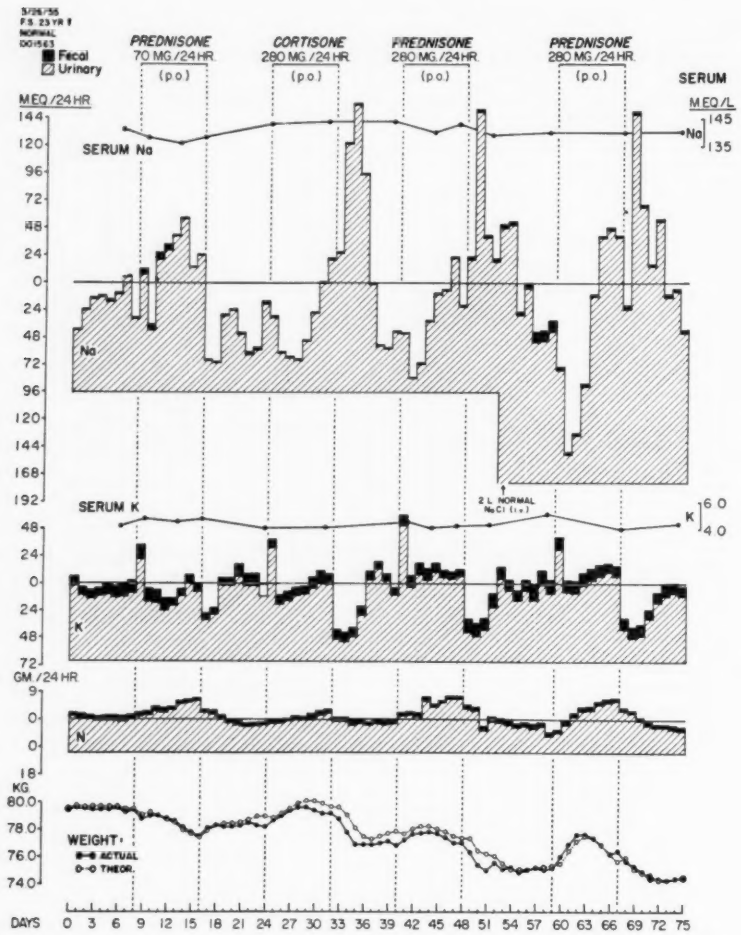

FIG. 8. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON SODIUM, POTASSIUM AND NITROGEN BALANCES, SERUM SODIUM, POTASSIUM AND ACTUAL AND "THEORETICAL" BODY WEIGHT IN F. S.

more than four times that of the parent compounds, the factor (12) estimated from anti-inflammatory effects in rheumatoid arthritis. Indeed, the mean ratio of potency of 1,4-diene to parent steroids based upon these studies on nitrogen metabolism is about eight. This is somewhat lower than the ratio (about 12) arrived at from studies on Addisonian patients (*2*).

In spite of the fluctuations in apparent calcium balance previously alluded to (and ascribed to the constipating effect of the steriods), it is evident from the magnitude of the increases of urinary calcium seen with the 1,4-diene steroids at the higher doses that they represent loss of bone tissue.

The effect of these steroids on potassium metabolism appears to represent the summation of four independent phenomena. *a*) The transient loss of potassium on starting treatment, which precedes nitrogen loss and sodium retention, and the corresponding transient retention of potassium on stopping treatment, which precedes nitrogen retention and sodium loss (see, for example, Figure 8, Days 8 and 16, 40 and 48), has been attributed to the anti-inflammatory action of steroids

(13). *b*) When large losses of nitrogen were induced with the steroids, some loss of potassium also occurred (see, for example, Figure 6, Days 30 to 33). However, in no instance was the magnitude of the loss commensurate (10) with that of nitrogen. This strongly suggests that *c*) potassium without nitrogen was stored during steroid administration. As noted above, a similar discrepancy appeared in the phosphorus balance, and both may be attributable to glycogen formation. Finally, *d*) with halogenated steroids, in doses too small to affect nitrogen balance, large losses of potassium were seen when sodium was being retained, and large gains of potassium were seen when sodium was lost after the steroids were discontinued (see, for example, Figure 9). This phenomenon appears to result from steroid-induced potentiation of sodium-potassium exchange at the renal tubular level (14, 15).

This study confirms the impression gained from an earlier one (2) that the 1,4-diene steroids increase renal tubular sodium transport, as they produced transient sodium retention when given in large doses. The subsequent "escape," which we attribute to enhancement of glomerular filtration rate, occurred in all instances.

The suppression of adrenal cortical activity of the 1,4-diene steroids is apparent from the marked decrease in 17-ketosteroid excretion (Figure 10). With cortisol, at two to five times the dose, only transient decrease of 17-ketosteroid excretion occurred. The difference appears to be the resultant of two phenomena. At the doses employed, both prednisolone and cortisol probably suppress adrenocorticotropic hormone (ACTH) secretion equally effectively. Consequently, secretion of adrenal 19-carbon steroids, as well as that of 21-carbon steroids destined for degradation to 19-carbon steroids, is suppressed. The urinary 17-ketosteroids during therapy (in this female subject) presumably represent degradation products of the administered steroid. The low figures observed with the 1,4-diene steroids result both from the enhanced ability of these steroids to suppress the adrenal cortex and from their limited conversion to 17-ketosteroids, as compared to the parent steroids (16). The latter phenomenon is illustrated in Figure 11, which shows studies in a female Addisonian subject lacking endogenous sources of 17-ketosteroids.

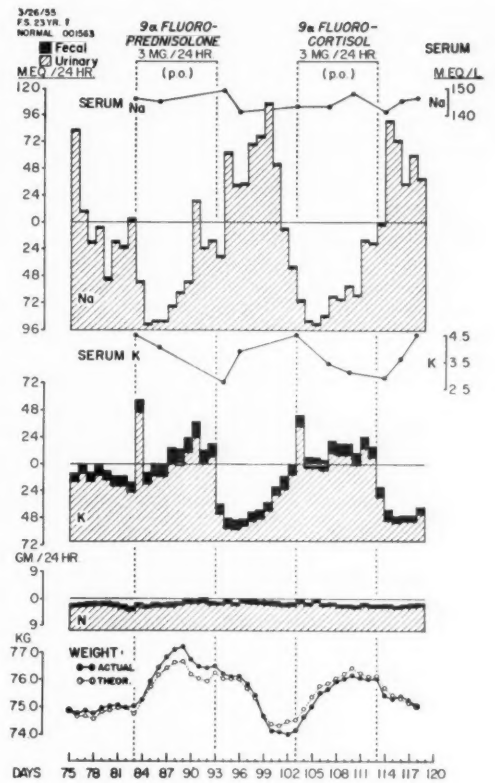

FIG. 9. THE EFFECT OF 9α-FLUOROPREDNISOLONE AND THE PARENT COMPOUND ON SODIUM, POTASSIUM AND NITROGEN BALANCES, SERUM SODIUM, POTASSIUM AND ACTUAL AND "THEORETICAL" BODY WEIGHT IN F. S.

SUMMARY

The effect of unsaturation by 1,2-dehydrogenation on the activity of 21-carbon steroids, given in moderate and very large doses, was explored with balance studies and steroid analyses in three subjects with normal adrenal cortical function. Unsaturation at carbon 1–2 results in an increase of anti-anabolic or catabolic activity which, judged from nitrogen loss, was some eight times that of the parent steroids. In the large doses, the unsaturated steroids produced sodium loss only after transient sodium retention. Unsaturation enhanced the ability of the parent compounds to suppress urinary 17-ketosteroids, decreasing their susceptibility to oxidative cleavage to 17-ketosteroids.

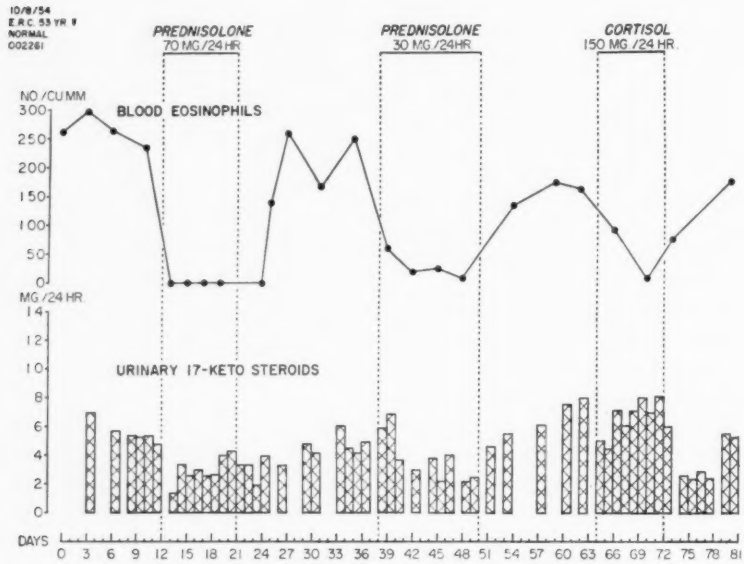

FIG. 10. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUNDS ON CIRCULATING BLOOD EOSINOPHILES AND URINARY 17-KETOSTEROIDS IN E. C.

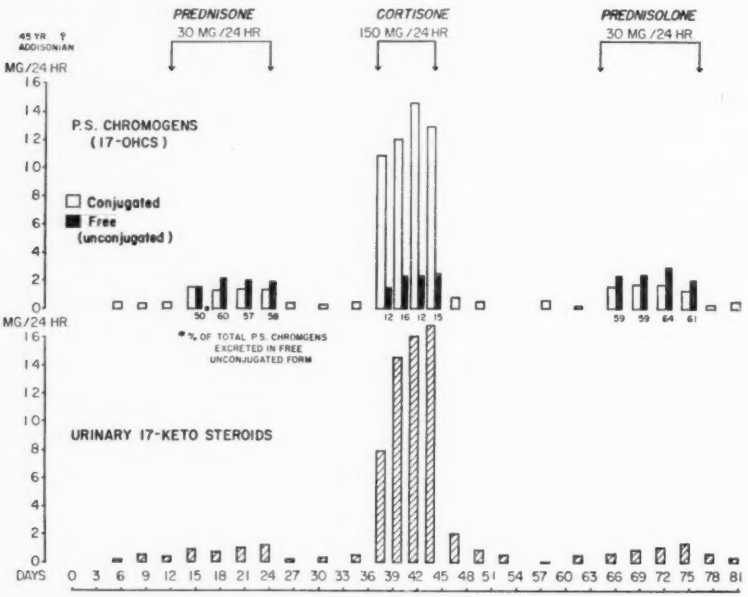

FIG. 11. THE EFFECT OF 1,4-DIENE STEROIDS AND THE PARENT COMPOUND ON URINARY FREE AND CONJUGATED PHENYLHYDRAZINE-SULFURIC ACID CHROMOGENS AND ON URINARY 17-KETOSTEROIDS IN A PATIENT WITH ADDISON'S DISEASE

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POTASSIUM TRANSPORT IN THE ACETYLCHOLINESTERASE-DEFICIENT ERYTHROCYTES OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) *

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The mechanism by which certain mammalian red cells accumulate potassium has been the subject of extensive investigation. Maintenance of a high intra-erythrocytic potassium concentration against a relatively low plasma concentration has been attributed to an active transport system. The precise relationship of such a transport system to cellular metabolism in general is obscure. Glycolysis, an important energy-yielding process in erythrocytes, has been held responsible for potassium reaccumulation and sodium extrusion by cold-stored human red blood cells when rewarmed to 37.5° (1, 2). Certain metabolic poisons which inhibit glycolysis also affect cation transport in red blood cells (3). These observations support the concept that the active transport mechanism may be geared to and utilize energy from glycolysis. Yet the hypothesis of an active transport system in human erythrocytes has never been absolutely established.

Some investigators have attempted to incriminate a single enzyme, acetylcholinesterase, as responsible for maintaining normal permeability in human and canine erythrocytes (4–6). These workers noted a retarding effect of acetylcholine on cation movement and on hemolysis and a reversal of this effect by physostigmine. These reports stimulated much interest in the possible role of acetylcholinesterase in governing sodium and potassium transport in erythrocytes. Other investigators, however, were unable to find evidence of a significant relationship between erythrocyte acetycholinesterase activity and potassium movement (7–10).

Recently we reported that the acetylcholinesterase activity of erythrocytes of eight patients with paroxysmal nocturnal hemoglobinuria (PNH)[1] was strikingly reduced (11, 12). Whether this defective cholinesterase activity is due to a deficiency of the enzyme *per se* or to the action of inhibitors has not been settled. Nevertheless, the PNH red cell provided an excellent experimental tool for investigating the role played by this enzyme in maintaining the potassium gradient of the human red cell. If the acetylcholinesterase enzyme plays a major role in potassium transport, the cholinesterase-deficient PNH cell should show alterations in potassium movement.

MATERIALS AND METHODS

Patient material. The clinical studies and detailed laboratory investigations regarding the finding of decreased erythrocyte acetylcholinesterase in PNH patients have been documented (11, 12). Case 1 of the current manuscript corresponds to Patient 6 of the previous publications and Case 2 to Patient 4.

Collection of blood. Sixty to 80 ml. of blood were collected from an antecubital vein into silicone-treated tubes containing heparin.[2] Saftidonor® [3] sets were employed for the collection of the blood.

Determination of acetylcholinesterase activity. A small portion of the collected blood was centrifuged at high speed (10,000 × G) for 10 minutes to pack the red cells. Following meticulous removal of the plasma, the red cells were washed three times with large volumes of 0.85 per cent sodium chloride solution and finally packed again by centrifugation at 3,000 rpm for five minutes.

Acetylcholinesterase activity of 0.2 ml. portions of

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[1] The abbreviation PNH will be used throughout the manuscript.

[2] Heparin sodium, 1,000 U.S.P. units (10 mg.) per ml., was kindly supplied by the Upjohn Company, Kalamazoo, Mich.

[3] We are indebted to Dr. E. B. McQuarrie, Biochemical Research Division, Cutter Laboratories, Berkeley, Cal., for the Saftidonor® sets.

washed, packed cells was determined manometrically using the Warburg technique. Measurements were carried out at 37.5° in a Ringer-Krebs bicarbonate medium. The final volume was 2.0 ml. Acetylcholine bromide in a final concentration of 5×10^{-3} M was employed as the substrate. The substrate had been recrystallized several times from absolute alcohol. The vessels were gassed with a mixture of 95 per cent N_2 and 5 per cent CO_2 for 10 minutes. The substrates and cells were mixed and allowed exactly three minutes to reequilibrate. Successive manometric readings were made at intervals of 10 minutes for one-half hour. The first 10 minute interval reading was considered the most accurate. This value was multiplied by six to give the rate per hour. Although a volume measurement was used for the determinations, the final expression of enzyme activity was in terms of dry weight (12).

Preparation of blood for influx and efflux studies. Following removal of the plasma, packed red cells were washed five times with Ringer-Krebs buffer.

For influx studies 6.0 ml. of washed, packed cells was mixed with 24.0 ml. of Ringer-Krebs buffer or of the subject's own heparinized plasma. (Similar results were obtained whether Ringer-Krebs buffer or the subject's own plasma were used as the diluent.) Small amounts of radioactive $K^{42}Cl$ were added to both the normal and PNH specimens simultaneously. The suspensions were mixed well and allowed to incubate at 37.5° in a Warburg bath with constant slow agitation.

The efflux studies were performed under the following experimental conditions: 8.0 ml. of washed, packed cells, 8.0 ml. of heparinized plasma, and $K^{42}Cl$ were mixed and allowed to incubate with slow, continuous agitation for four hours in silicone-treated Erlenmeyer flasks. The labeled cells were separated by centrifugation and washed thrice with Ringer-Krebs buffer (pH 7.4). To 6.0 ml. of these washed cells was added 24.0 ml. of Ringer-Krebs buffer. The suspensions were mixed and incubated at 37.5° in a Warburg bath.

Sampling of aliquots for influx studies. Four ml. samples were removed immediately and at 30 to 60 minute intervals over a four hour period following the addition of the isotope to the reconstituted mixtures. The samples were centrifuged for five minutes at 3,000 rpm to separate cells from plasma. One ml. of plasma was plated in a planchette, baked dry, and the radioactivity determined in a Geiger counter. To another 1 ml. aliquot of plasma 7.0 ml. of water was added and the protein precipitated by the addition of 2.0 ml. of 25 per cent trichloracetic acid. The filtrate was analyzed for potassium by employing a Beckman spectrophotometer model DU with a flame attachment. One-half ml. of red cells was hemolyzed by the addition of 4.5 ml. of water. One ml. of the hemolysate was plated in a planchette, dried, and the radioactivity determined. All counts were corrected for decay and background. The remaining hemolysate was rendered protein-free by the addition of trichloracetic acid. The filtrate was analyzed for potassium.

Sampling of aliquots for efflux experiments. Four ml. samples were removed from the reconstituted mixture (labeled cells and Ringer-Krebs buffer) at 30 to 60 minute intervals over a four hour period. Potassium analysis and radioactivity determinations were performed on the supernatant and on the cells by the methods described for the influx studies.

Mathematical treatment of the data. The treatment of the data is abstracted from that of Solomon (13). The following equation governs or describes these experiments:

$$\frac{dp}{dt} = -k_{ab}p + k_{ba}q \qquad 1)$$

where p = plasma concentration of K^{42}, q = red cell concentration of K^{42}, k_{ab} and k_{ba} = transfer coefficients (k_{ab} from plasma to cells and k_{ba} from cells to plasma). Multiplying this equation through by $\frac{v_p}{v_q}$, one obtains the following:

$$\frac{dP}{v_q dt} = -k_{ab}\left(\frac{v_p}{v_q}\right)p + k_{ba}\left(\frac{v_p}{v_q}\right)q \qquad 2)$$

where P is the amount of K^{42} in the plasma, and v_p and v_q are the volumes of plasma and cells, respectively. This equation gives the amount of P transported per unit time per unit volume of cells, and this quantity is independent of v_p and v_q. Therefore, $k_{ab}\left(\frac{v_p}{v_q}\right)$ and $k_{ba}\left(\frac{v_p}{v_q}\right)$ are the constants which are independent of the v_p and v_q. The efflux equations are of the same form as the influx equations.

RESULTS

Acetylcholinesterase activity of normal and PNH erythrocytes

Table I shows the acetylcholinesterase activity of the PNH red cells compared to the mean value of determinations on the cells of 20 normal subjects. Under these experimental conditions the erythrocytes of Case 1 possessed no enzyme activity. The activity of red cells of Case 2 was 17.3 per cent of normal.

TABLE I

Acetylcholinesterase activity of normal and paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes

	Manometric data: $\mu L.$ CO_2 per mg. dry weight per hour	% of normal
Normal (average of 20)	15.0	
Case 1	0	0
Case 2	2.6	17.3

Potassium transport data

The data obtained from two normal subjects and the two PNH patients are plotted in Figures 1 and 2. Each figure is typical of four separate experiments. It can be seen that potassium transport, which is characterized by the quantities k_{ab} (v_p/v_q) and k_{ba} (v_p/v_q) in the case of the influx studies, is virtually the same for normal and PNH cells. The computed transfer coefficients are in good agreement with those reported by Solomon (13).

The precision of the efflux experiments was not very high. Nevertheless, the curves obtained from the data appeared to have the same slopes, indicating that there is no difference in the transfer coefficients of normal and PNH cells.

DISCUSSION

Several investigators have suggested that specific acetylcholinesterase, which is situated entirely in the cell membrane, is concerned with the permeability of the red cell (4–6). When acetylcholine was added to a suspension of red cells in an isotonic bicarbonate medium, it not only reduced the loss of potassium but also delayed hemolysis. In an isotonic sodium chloride or sodium bicarbonate medium containing 35 mM of KCl per L. at pH 8.0, the addition of acetylcholine produced a small increase (5 to 10 per cent) in cellular potassium. Physostigmine, an inhibitor of acetylcholinesterase, reversed or blocked the above effects. When benzoylcholine, a substrate not appreciably hydrolyzed by acetylcholinesterase, was substituted for acetylcholine, such changes likewise did not occur. It was concluded from these studies that cholinesterase activity is responsible in some manner for maintenance of normal potassium and sodium concentrations in human red cells (4–6).

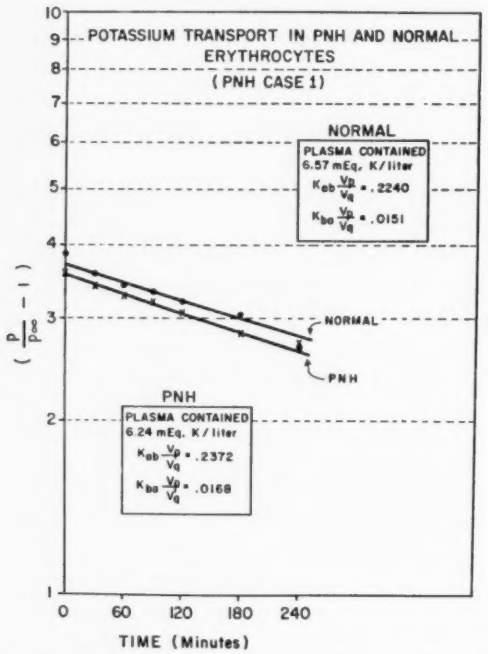

FIG. 1. POTASSIUM TRANSPORT IN PNH AND NORMAL ERYTHROCYTES (PNH CASE 1)

Parpart and Hoffman (7) repeated the experiments of Lindvig, Greig and Peterson (6). The addition of acetic acid to red cell suspensions brought about the same results as the addition of acetylcholine with respect to retarding both cellular loss of potassium and hemolysis. They concluded that the effect of acetylcholine was due to the reduction in pH produced by the addition of this substrate. Physostigmine failed to block the effect of acetic acid but did abolish the acetylcholine effect, further evidence in favor of their interpretation. These authors concluded that there was no evidence linking cation permeability of human red cells and the activity of the acetylcholinesterase system.

Taylor, Weller and Hastings studied the effect of inhibitors of cholinesterase (physostigmine, diisopropyl fluorophosphate) on the potassium concentration gradient as well as on the rate of potassium exchange of human erythrocytes (8). Cholinesterase inhibitors brought about a loss of potassium from the cells primarily by decreasing the rate at which potassium entered the cell from the plasma. No direct relationship between inhibition of the enzyme and the rate at which potassium left the cell was found. A loss of cellular potassium was noted only when concentrations of the inhibitors were at least 100 times that necessary to inactivate the enzyme.

Goodman, Marrone and Squire found no evidence of a relationship between erythrocyte acetylcholinesterase activity and the *in vivo* potassium gradient (9). Recently Gale, Brown and Eadie have demonstrated that inhibition of red cell cholinesterase with and without the addition of acetyl-

choline did not cause potassium loss from cells at 4° or at 37.5° (10). Re-entry of potassium at 37.5° in cells previously stored at 4° was also unaffected.

Our experiments revealed that human erythrocytes deficient in cholinesterase activity because of a disease state (PNH) were still capable of maintaining a normal potassium concentration gradient. The inward and outward potassium rate constants were similar for both PNH and normal erythrocytes.

The acetylcholinesterase activity of red cells of Case 2 was approximately 17 per cent of normal. Bullock, Grundfest, Nachmansohn and Rothenberg noted that a nerve may still transmit impulses even when as much as 80 per cent of the enzyme activity is inhibited (14). This suggests the possibility that the residual acetylcholinesterase activity in the PNH erythrocytes of Case 2 might account for the normal transfer coefficients. On the other hand, the red cells of Case 1 possessed *no enzyme activity* when determined by the manometric method. Yet potassium influx and efflux were not significantly altered. These observations are not compatible with the concept that small amounts of the enzyme can account for the maintenance of a high intracellular potassium concentration. It remains possible that other methods of determining erythrocyte acetylcholinesterase might have revealed traces of enzyme activity in the cells of Case 1. On the other hand, Holland and Greig demonstrated that only 60 per cent inhibition of acetylcholinesterase activity was necessary to increase the permeability of the canine erythrocyte membrane (5).

Cation transport in sickle cell anemia cells has been investigated (15). Marked changes in cation transport occurred when the transformation of the disc to the sickle shape took place. These marked changes in cation transport were not accompanied by significant alteration in acetylcholinesterase activity. Similar observations were made in our laboratory during the current studies.

Hellem and Skaug demonstrated decreased penetration of P^{32} through the PNH erythrocyte membrane (16). Whether this is related to the deficiency of acetylcholinesterase activity remains unsettled. These authors concluded that the decreased permeability to P^{32} might be due to membrane abnormalities or to altered metabolic processes in the interior of the PNH erythrocyte.

The blood platelet is another formed element bathed in the same fluid (plasma) as the red cell. Recent studies have demonstrated that like the human red cell, the human platelet contains a high concentration of potassium and a relatively low sodium concentration (17). Rather convincing histochemical and manometric studies have demonstrated that normal human platelets contain no measurable cholinesterase (18). These observations suggest that despite the absence of acetylcholinesterase activity, the platelet is capable of maintaining a high internal potassium concentration. In a similar vein the apparent lack of acetylcholinesterase in the erythrocytes of some mammalian species has led certain investigators to conclude that the enzyme cannot play a universal role in maintaining cell permeability (18).

The acetylcholinesterase-deficient PNH red cell provided an excellent opportunity to test the hypothesis that this enzyme is responsible for maintaining normal permeability of the human erythro-

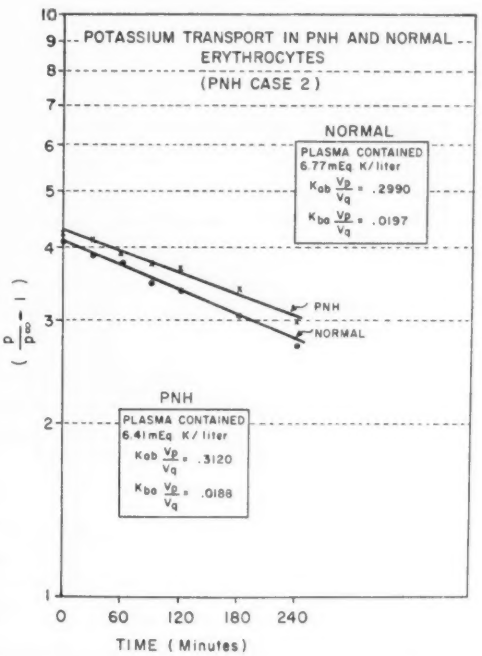

FIG. 2. POTASSIUM TRANSPORT IN PNH AND NORMAL ERYTHROCYTES (PNH CASE 2)

cyte. It was not necessary to inhibit acetylcholinesterase activity by drugs (e.g., physostigmine) which in turn might have produced nonspecific effects. That the PNH cell still showed normal potassium influx and efflux rates provides evidence against the above hypothesis. It remains possible that the acetylcholinesterase enzyme may play some role in permeability of normal human erythrocytes. Nevertheless, the present study indicates that this enzyme is *not essential* for normal permeability in human erythrocytes.

SUMMARY

The influx and efflux rates of potassium in normal human and PNH (paroxysmal nocturnal hemoglobinuria) erythrocytes have been investigated. PNH erythrocytes markedly deficient in acetylcholinesterase activity still had normal potassium influx and efflux rates. The computed transfer coefficients showed virtually no difference. These findings suggest that erythrocyte acetlycholinesterase activity is not essential for the maintenance of normal human red cell permeability.

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METABOLISM OF NORADRENALINE IN THE HUMAN *

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It is now generally accepted that noradrenaline is the neurohormone of the sympathetic nerves (1–4) and is released as such at the nerve endings. However, it is not understood how the body metabolizes noradrenaline. This paper describes a method for separating and identifying the various metabolic products of noradrenaline in the human and discusses the import of this procedure as a tool for studying those clinical entities which represent an aberration in the physiology of the sympathetic nervous system.

As early as 1895 Cybulski (5) showed that extracts of the adrenal medulla, when injected intravenously, increased the pressor activity of the urine. Later Holtz, Credner, Kroneberg and Shümann (6, 7) and von Euler and Hellner (8) demonstrated that this pressor activity was due to adrenaline, noradrenaline, and, to a far lesser extent, hydroxytyramine. Subsequently, von Euler, Luft and Sundin (9) showed that by infusing adrenaline, 0.4 to 1.7 per cent of the infused adrenaline appeared in the urine, and, in similar experiments performed with noradrenaline (10), 1.5 to 3.3 per cent of the infused noradrenaline was excreted in the urine. This therefore means that over 95 per cent of the adrenaline and noradrenaline is metabolized following release; consequently, the metabolic products which appear in the urine must be in a relatively biologically inactive form.

The first of these relatively inactive metabolic products was demonstrated in 1940 by Richter (11). He showed that by ingesting large quantities of adrenaline, a conjugate of adrenaline could be isolated from the urine. He concluded that this conjugate was a sulfate. Beyer and Shapiro (12) performed somewhat similar experiments but concluded that the conjugate was a glucuronide. Dodgson, Garton and Williams in 1947 (13) fed rabbits d-adrenaline and isolated from the urine of these rabbits a conjugate of adrenaline. Clark and Drell (14), repeating this work, showed that rabbits metabolized dl- and l-adrenaline in a similar manner and that the conjugate was a glucuronide.

Then in 1952 and 1953 Schayer, Smiley, Kaplan and Kennedy (15–17) published their papers on the metabolism of adrenaline. They not only showed something about the cleavage of adrenaline but pointed out that there were at least five or six urinary catabolites of adrenaline. In 1957 Armstrong, McMillan and Shaw (18, 19) identified 3-methoxy-4-hydroxymandelic acid as a major urinary metabolite of adrenaline in the human. Subsequently, Axelrod and associates (20, 21) isolated metadrenaline (3-O-methyladrenaline) and normetadrenaline (3-O-methylnoradrenaline) from rat urine and metadrenaline from human urine. Also in 1957 von Euler (22) demonstrated 3,4-dihydroxymandelic acid in human urine, and in 1958 LaBrosse, Axelrod and Sjoerdsma (23) isolated normetadrenaline from the urine of pheochromocytoma patients. Goodall, Rosen and Kirshner (24, 25) infused normal male subjects with labeled dl-adrenaline-2-C^{14} and separated and identified a number of the urinary catabolites of adrenaline. Hitherto, with the exception of 3-methoxy-4-hydroxymandelic acid, none of the metabolic products of noradrenaline in the normal human have been separated or identified.

MATERIALS

A. dl-Noradrenaline-2-C^{14}, specific activity 20 mc. per mM, from Volk Radiochemical Company, Chicago, Ill. Prepared by the method of Howton, Mead and Clark (26).

B. Amberlite IRC-50, X E-64 from Rohm and Haas Company, Philadelphia, Pa.

C. Dowex 1-2 X, 200–400 mesh from Dow Chemical Company, Midlands, Mich.

* Supported by United States Public Health Service Grant H-4309.

† Established Investigator of the American Heart Association.

METHODS

A. Infusion of dl-noradrenaline-2-C¹⁴. Six healthy adult males between the ages of 20 and 35 years were infused with 9.5 μc. (80 μg. of free base) of sterile dl-noradrenaline-2-C¹⁴. The noradrenaline was diluted with 250 ml. of physiological saline and infused over a period of one hour. Urine was collected after the infusion period, at one hour intervals for the next five hours, and then every six hours for the next 18 hours, thereby covering a total collection period of 24 hours. The urine specimens were refrigerated immediately after collection and stored at −15°.

Aliquots of each hourly specimen were pooled to give a six hour sample, and aliquots of each six hour sample were pooled to give a 24 hour sample. The radioactivity in all specimens was determined with a thin window geiger tube (18 cpm background) and corrected to infinite thinness by reference to a previously prepared absorption curve.

B. Separation of urinary metabolic products of noradrenaline. The principle behind this method is the absorption of the basic catabolites on Amberlite IRC-50, the absorption of acidic catabolites on Dowex 1-2X and the passage of the neutral catabolites through both resins.

An aliquot of urine (5 to 10 ml.) containing 15,000 to 30,000 cpm is passed through a 3 × 1 cm. column of Amberlite IRC-50 prepared as previously described (27). The column is then washed with 15 ml. of water. The effluent and wash are combined and saved. The IRC-50 column is then eluted with 20 ml. of 0.5 N acetic acid. The eluate is evaporated to dryness and the residue dissolved in 0.5 ml. of water. This solution is then chromatographed for 15 to 18 hours on Whatman No. 1 filter paper using *n*-butanol saturated with N HCl as the solvent. After drying, the paper is cut into 1 cm. strips; each of the strips is eluted with water and an aliquot plated out for the determination of radioactivity. Three radioactive peaks are found.

The effluent and wash from the IRC-50 column is then placed on a 10 × 1 cm. column of Dowex-1 acetate buffered at pH 6.1. This column is then washed with 30 ml. of water; again the effluent and wash are collected and assayed for radioactivity.

The Dowex-1 column is then attached to an automatic fraction collector and eluted with 0.05 M ammonium acetate, pH 4.8. Three ml. fractions are collected. The course of the fractionation is followed by assaying the radioactivity of every third sample. When the radioactivity of the eluate returns to background (usually after 75 to 80 ml.) the elution is continued with 50 ml. of 0.3 M ammonium acetate, pH 4.8. This is again repeated with 100 ml. of 1.0 M and with 150 ml. of 3.0 M ammonium acetate, pH 4.8. Each elution yielded one radioactive peak except the 3.0 M buffer which yielded two peaks.

RESULTS

A. IRC-50 elution

Three radioactive peaks were eluted from the paper chromatograms. They represent basic compounds. One peak was chromatographically identical to normetadrenaline and another peak to free noradrenaline. The third peak has not yet been

TABLE I

Urinary excretion of noradrenaline and its metabolic products in the 24 hours following an infusion of dl-noradrenaline-2-C¹⁴ *

Subjects	% Infused dose recovered	IRC-50 fractions				Dowex-1 fractions					Dowex effluent	% Recovery urinary-C¹⁴
		Total	Nor†	NMA‡	Unk§	0.05 M eluate NMAX‖	0.30 M eluate Unk	1.0 M eluate MOMA¶	3.0 M(A) eluate DOMA** +Unk	3.0 M(B) eluate DOMA +Unk		
M. A.	62	10	4	3	2	17	9	30	8	16	3	93
S. N.	69	8	2	2	3	20	10	33	12	12	4	99
L. O.	64	12	4	3	3	21	7	38	11	9	5	103
B. O.	72	9	4	3	2	22	13	30	10	11	4	99
W. E.	65	11	3	4	2	20	10	30	12	14	5	102
M. R.	67	9	4	2	2	16	8	29	12	13	3	90
Average	67	10	4	3	2	19	10	32	11	13	4	98
S. D.††	4	2	1	1	1	2	2	3	2	2	1	5
S. E.‡‡	2	1	1	1	1	1	1	1	1	1	1	2

* Figures are expressed as per cent of total urinary radioactivity except Column 1, which is the per cent of the infused dose recovered in the urine.
† Nor, noradrenaline.
‡ NMA, normetadrenaline.
§ Unk, unknown.
‖ NMAX, normetadrenaline conjugate.
¶ MOMA, 3-methoxy-4-hydroxymandelic acid.
** DOMA, 3,4-dihydroxymandelic acid.
†† S. D., standard deviation.
‡‡ S. E., standard error of the mean.

identified and represents only 2 per cent of the total radioactivity found in the 24 hour urine sample (see Table I).

B. Dowex-1 elution

1. 0.05 M eluate. This fraction is an acid labile conjugate of normetadrenaline. After heating for 90 minutes with 0.1 N hydrochloric acid at 100°, 82 per cent of the radioactivity originally present is recovered as normetadrenaline. The normetadrenaline was isolated from the hydrolysate by absorption on IRC-50 and identified by paper chromatography using two different solvent systems, *i.e.*, butanol saturated with N HCl and isopropanol-ammonia-water (8–1–1). Because of its ready hydrolysis in dilute acid and since it is hydrolyzed to about 70 per cent by Mylase P® (Nutritional Biochemicals), an enzyme preparation containing arylsulfatase, the conjugate is probably a sulphate ester. The conjugate is not hydrolyzed after a 24 hour incubation with either bacterial (Sigma) or animal (Warner-Chilcott) β-glucuronidase preparations.

2. 0.3 M eluate. The radioactivity in this fraction appears to be due to a single compound since only one radioactive area can be detected by paper chromatography using butanol-N HCl and isopropanol-ammonia-water as solvents. The compound, which has not yet been identified, represents 10 per cent of the total radioactivity in the 24 hour urine (see Table I).

3. 1.0 M eluate. The radioactivity in this fraction is chromatographically identical with 3-methoxy-4-hydroxymandelic acid (see Table I). Again two different solvent systems were used for identification, *i.e.*, butanol-N HCl and isopropanol-ammonia-water.

4. 3.0 M eluate. Two rather diffuse peaks are obtained from the Dowex column in the 3.0 M elution. Upon chromatographing these peaks in butanol-N HCl, butanol-acetic acid-water (4–1–1) and in isopropanol-ammonia-water, two radioactive areas are obtained from each of these fractions. One of the radioactive areas in each of these fractions corresponds to 3,4-dihydroxymandelic acid; the other radioactive areas have not been identified. Approximately 30 to 50 per cent of the radioactivity eluted from the filter paper is found in the fraction tentatively identified as 3,4-dihydroxymandelic acid.

C. General results

It should be noted that only 4 per cent of the total radioactivity found in the 24 hour urine passed through both columns, and too, there was no detectable radioactivity left on either the IRC-50 or the Dowex-1 column.

When dl-noradrenaline-2-C^{14} is infused, 67 ± 4 per cent of the radioactivity is recovered from the urine within a period of 24 hours (see Table 1). Figure 1 shows, in six subjects, the average hourly excretion of radioactivity. These findings compare well with the 73 ± 2 per cent recovery of radioactivity in the adrenaline infusion experiments previously reported from this laboratory (24) and the adrenaline infusion experiments of Resnick and Elmadjian (28). Table I presents the data on the excretion of noradrenaline and the catabolites of noradrenaline over a 24 hour period following an infusion of dl-noradrenaline-2-C^{14}. From Table I it is apparent that approximately 85 per cent of the radioactivity noted in the urine can be separated on Dowex-1 and about 10 per cent on IRC-50. The identified noradrenaline catabolites eluted from the Dowex-1 are: normetadrenaline conjugate; 3-methoxy-4-hydroxymandelic acid; 3,4-dihydroxymandelic acid. Of these products, 3-methoxy-4-hydroxymandelic acid is the largest single fraction, representing about 32 per cent of the total radioactivity. The second largest fraction is the normetadrenaline conjugate represent-

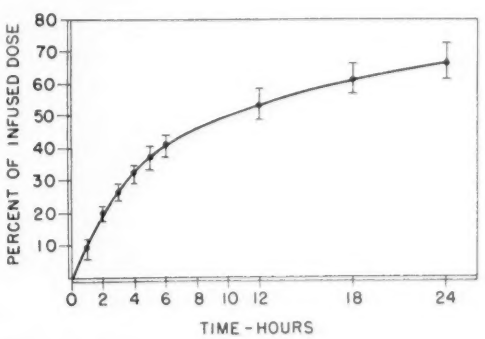

FIG. 1. EXCRETION OF RADIOACTIVITY IN THE URINE AFTER INFUSIONS OF NORADRENALINE-2-C^{14}

The points represent the averages of six subjects; the vertical lines represent the range of variations.

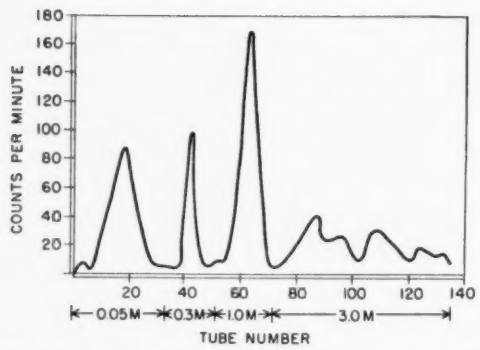

FIG. 2. SEPARATION OF URINARY METABOLITES OF NORADRENALINE-2-C^{14} ON DOWEX-1

The concentration at the bottom of the graph refers to the ammonium acetate buffers.

ing 19 per cent; the remaining 35 per cent eluted from the Dowex column is divided about equally among the other three peaks (0.3 M and 3.0 M eluates) which include 3,4-dihydroxymandelic acid and two or three unidentified compounds. Figure 2 graphically represents the radioactivity in the noradrenaline catabolites eluted from Dowex-1 from a 24 hour urine sample.

Ten per cent of the total radioactivity is absorbed on IRC-50. Of this radioactivity, 4 per cent is due to unchanged noradrenaline, 3 per cent to free normetadrenaline and 2 per cent to an unidentified compound. Previous work (10) on the excretion of noradrenaline in urine following an infusion showed 1.5 to 3.3 per cent of the infused dose appearing in the urine as free noradrenaline and/or conjugates of noradrenaline.

Table II shows the hourly excretion pattern of noradrenaline and its metabolic products. Though this table represents the pattern from a single subject, this pattern is similar to that of the five other subjects. From Table II it is also apparent that the noradrenaline is rapidly metabolized by the body. This is seen by the rapid disappearance of free noradrenaline from the urine after the infusion period. Approximately 16 per cent of the radioactivity noted in the urine during the infusion period is free noradrenaline. This decreases to 3 per cent during the second hour and 1 per cent during the third and fourth hours; thereafter only small amounts of radioactive noradrenaline can be detected. The normetadrenaline follows a similar pattern as does the unidentified

TABLE II

*Excretion of noradrenaline and its metabolic products during the 24 hour period following an infusion of dl-noradrenaline-2-C^{14} **

Time in hours	% Infused dose recovered	IRC-50 fractions				Dowex-1 fractions						
		Total	Nor†	NMA	Unk	0.05 M eluate NMAX	0.30 M eluate Unk	1.0 M eluate MOMA	3.0 M(A) eluate DOMA +Unk	3.0 M(B) eluate DOMA +Unk	Dowex effluent	% Recovery urinary-C^{14}
1	11	38	16	12	8	10	2	33	4	8	4	86
2	10	9	3	4	1	15	7	41	11	9	5	98
3	7	3	1	1	0.4	24	12	37	8	19	3	107
4	6	3	1	1	0.4	26	11	30	8	21	4	102
5	5	2	0.7	0.7	0.5	21	8	29	12	22	4	97
6	4	2	0.9	0.7	0	26	7	22	19	17	4	98
6–12	10	2	Trace	0	0	33	15	16	12	17	3	98
12–18	7	1	0	0	0	33	11	18	13	20	3	99
18–24	5	2	0	0	0	19	8	16	12	15	11	83
Calculated 24 hour aliquot	65	9	4	3	2	22	9	28	9	15	4	96
24 hour aliquot experimental	65	11	3	4	2	20	10	30	12	14	5	103

* Figures are expressed as per cent of total urinary radioactivity except Column 1, which is the per cent of the infused dose recovered in the urine.

† For abbreviations, see Table I.

TABLE III

Excretion of noradrenaline and its metabolic products during the infusion period and at the end of six hours and at the end of 24 hours following the infusion of dl-noradrenaline-2-C^{14} *

Time	% Infused dose recovered	IRC-50 fractions				Dowex-1 fractions						% Recovery urinary-C^{14}
		Total	Nor†	NMA	Unk	0.05 M eluate NMAX	0.30 M eluate Unk	1.0 M eluate MOMA	3.0 M(A) eluate DOMA +Unk	3.0 M(B) eluate DOMA +Unk	Dowex effluent	
Infusion	10±2	37±3	17±3	10±3	8±3	9±1	2±4	32±3	4±1	6±1	3±1	93±7
6 hours	41±4	12±1	5±1	4±1	2±1	17±2	8±2	36±5	9±1	11±2	5±1	98±6
24 hours	67±4	10±2	4±1	3±1	2±1	19±2	10±2	32±3	11±2	13±4	4±1	98±5

* Figures are expressed as per cent of total urinary radioactivity except Column 1, which is the per cent of the infused dose recovered in the urine.

† For abbreviations, see Table I.

compound eluted from the IRC-50. The amounts of these later catabolites were less than that of free noradrenaline.

From the Dowex-1 elution shown in Table II, it is apparent that 3-methoxy-4-hydroxymandelic acid represents the largest single catabolite and that the radioactivity in this fraction is initially high (33 per cent, increasing to 41 per cent in the second hour and gradually decreasing during the remaining 22 hours). On the other hand, the radioactivity in the normetadrenaline conjugate fraction gradually increased from an initial 10 per cent during the infusion period to 33 per cent during the 12 to 18 hour collection period. It should be noted that the 18 to 24 hour sample had to be concentrated four times to obtain sufficient radioactivity for assay purposes. Because of the large amount of salt present in this concentrated sample, a distorted fractionation picture was obtained, thereby leading one to believe that most of the radioactivity in the Dowex-1 effluent of the 18 to 24 hour sample is probably due to the normetadrenaline conjugate.

The unknown catabolite resulting from the 0.3 M eluate appears to increase for two hours following the infusion and then plateaus at a constant excretion rate. A somewhat similar pattern is noted for each of the 3.0 M eluates.

A comparison was made between the 24 hour distribution of C^{14} calculated from each hourly sample and that actually obtained by measuring the distribution of radioactivity in a 24 hour aliquot (see Table II). Good agreement was obtained which indicates the accuracy of this procedure.

Table III compares the distribution of radioactivity in the various catabolites of noradrenaline at the end of the infusion period, at the end of six hours, and at the end of 24 hours. Total recoveries were better in the six and 24 hour periods than in the infusion period. A marked difference can be seen in the distribution of radioactivity at the end of the infusion period and at the end of six and 24 hours. There was only a slight difference between the six and 24 hour sample.

A total of 10 radioactive fractions are obtained by using a combination of ion exchange and filter paper chromatography to separate the metabolic products of noradrenaline-2-C^{14}. Five of these fractions have been identified. Of the remaining five, at least three are distinctly different compounds. These are: unidentified radioactive peak obtained upon chromatographing the IRC-50 eluate, the material eluted from the Dowex-1 column with 0.3 M ammonium acetate, and the unidentified compound eluted with 3.0 M ammonium acetate. Although dihydroxymandelic acid and an unidentified radioactive area are found upon chromatographing each of the peaks obtained in the 3.0 M elute, it is not certain that these unidentified compounds are different from each other. Because of the small amount of radioactivity in the Dowex effluent, an unequivocal chromatogram of this fraction has not yet been obtained. The work of Booth, Emerson, Jones and DeEds (29) suggests that one of these compounds may be *m*-hydroxymandelic acid, and it is quite possible that one may find glycine or glucuronidate conjugates of the previously identified compounds among the unidentified fractions.

DISCUSSION

After an infusion of dl-noradrenaline-2-C^{14}, 67 ± 4 per cent of the infused radioactivity is recovered from the urine in 24 hours (see Figure 1). The fact that the urinary excretion of radioactive catabolites continues for longer than 24 hours, and since only small amounts of free noradrenaline appear in the urine after the infusion, would imply that the noradrenaline *per se* rapidly disappears from the blood stream but that the metabolic products are gradually released into the blood stream for renal clearance. The fact that the radioactivity in the blood during and especially after the infusion was very low supports this concept.

Why the radioactive noradrenaline rapidly disappears from the urine and why the metabolic products continue to be excreted over a 24 hour period is not yet clear. However, it is quite possible that the tissue cells pick up the noradrenaline, store it as a complex (30, 31) and gradually release it for metabolism, thereby permitting the metabolic products to gradually appear in the general circulation for renal clearance. As an alternate explanation, the noradrenaline could be metabolized at the time of release and the metabolic products could diffuse into the tissue spaces or cells from which they could gradually diffuse back into the blood stream and be excreted by the kidney. Either of these processes would account for the rapid disappearance of free noradrenaline and the gradual excretion of the metabolic products. The fact that small amounts of free radioactive noradrenaline are excreted over the first 12 hours (see Table II) would indicate that at least some of the free noradrenaline is stored in the tissues for subsequent release and metabolism. Obviously, how noradrenaline is precisely metabolized cannot be shown until tissue studies are made following an injection of labeled noradrenaline. Such work is currently underway.

From the data in Table III, about 55 per cent of the metabolic products of noradrenaline appear in the urine as O-methylated compounds, namely normetadrenaline, normetadrenaline conjugate and 3-methoxy-4-hydroxymandelic acid. If one assumes that the compounds, other than the normetadrenaline conjugate, which are absorbed on the Dowex-1 column arise from oxidative deamination, then 65 per cent of the metabolic products are acidic compounds which probably result from the action of monoamine oxidase upon noradrenaline and normetadrenaline.

The major products of noradrenaline metabolism in man are 3-methoxy-4-hydroxymandelic acid and a conjugate of normetadrenaline. The former compound results from the action of the enzymes amine oxidase, aldehyde dehydrogenase and catechol-O-methyl transferase; the latter compound from the action of catechol-*o*-methyl transferase and the enzymes involved in the conjugation. Similarly, the major products of adrenaline metabolism in man (24, 25) are 3-methoxy-4-hydroxymandelic acid and a conjugate of metadrenaline.

The conclusion (32) that O-methylation precedes oxidative deamination and that the former process is the principal mechanism for the initial inactivation of adrenaline is not sufficiently supported by evidence. By an analysis of the 24 or 48 hour urine specimen one cannot determine the sequence of reaction in the formation of 3-methoxy-4-hydroxymandelic acid from adrenaline or noradrenaline. Data (33) on the metabolic products of dl-adrenaline-2-C^{14} which appear in the urine immediately after an infusion of adrenaline-2-C^{14} in normal subjects and subjects treated with an amine oxidase inhibitor (Marsilid®) show that, in the subjects who received Marsilid®, the radioactivity in the urinary adrenaline was twice that of the untreated subjects; the radioactivity in the metadrenaline and conjugated metadrenaline fraction was increased 65 per cent above the untreated subjects. These data suggest that, during the short period of time when adrenaline and noradrenaline have physiological activity, amine oxidase activity is of considerable importance in the inactivation of these hormones. The presence of significant amounts of 3,4-dihydroxymandelic acid in the urine and the recent reports by Pekkarinen, Castren and Iisolo (34) and Shore, Prockop, Spector and Brodie (35) that the noradrenaline levels in brain and other tissues were significantly elevated after treatment with an amine oxidase inhibitor (iproniazed, Marsilid®), also support this concept. The present evidence shows that oxidative deamination and O-methylation are the major alternative pathways for the metabolism of adrenaline and noradrenaline, and strongly sug-

gests that each contributes significantly in the initial inactivation of adrenaline and noradrenaline during the period of physiological activity.

The results reported here for dl-noradrenaline are not necessarily applicable to the natural occurring l-isomer. However, from the demonstration by Schayer and co-workers (15, 16) that both l-adrenaline and dl-adrenaline were metabolized similarly by the rat, and from the observations that both the l and d isomers of adrenaline serve almost equally well as substrates for amine oxidase (36) and catechol-O-methyl transferase (37), one is led to believe that the quantitative results obtained with dl-noradrenaline would be in very close agreement with those of the l-isomer.

Noradrenaline is the neurohormone of the sympathetic nerves (1–4). In view of this, the metabolic products of noradrenaline would represent a barometer of the functioning of the sympathetic nervous system. There are a number of clinical conditions which result from an aberration in the physiology of the sympathetic nervous system, *i.e.*, chronic postural hypotension, certain forms of neurogenic hypertension, diabetic neuropathy, sympathetic nerve tumor, and so forth. Therefore, the procedure herein described for separating and identifying the metabolic products of noradrenaline could be a useful tool for studying these conditions as well as evaluating their therapy.

SUMMARY

Six adult males were infused with labeled noradrenaline and the urinary catabolites were separated and identified. A procedure is herein described which utilizes a combination of filter paper and ion exchange chromatography to separate the urinary metabolic products of labeled noradrenaline. Sixty-seven ± 4 per cent of the infused radioactivity was recovered in the 24 hour urine. The total radioactivity which appeared in the urine was distributed among the various catabolites of noradrenaline as follows: noradrenaline, 4 ± 1 per cent; normetadrenaline, 3 ± 1 per cent; normetadrenaline conjugate, 19 ± 2 per cent; 3-methoxy-4-hydroxymandelic acid, 32 ± 3 per cent; 3,4-dihyroxymandelic acid, and five unidentified fractions, 40 per cent. The 3,4-dihydroxymandelic acid has been tentatively identified as part of two of the unidentified fractions.

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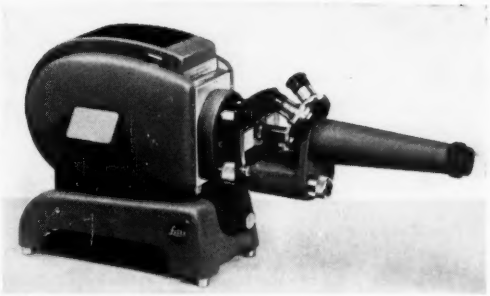

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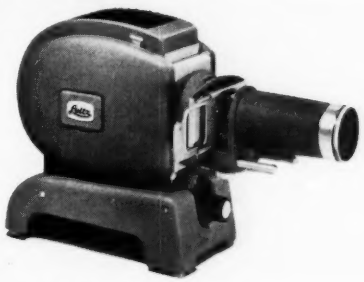

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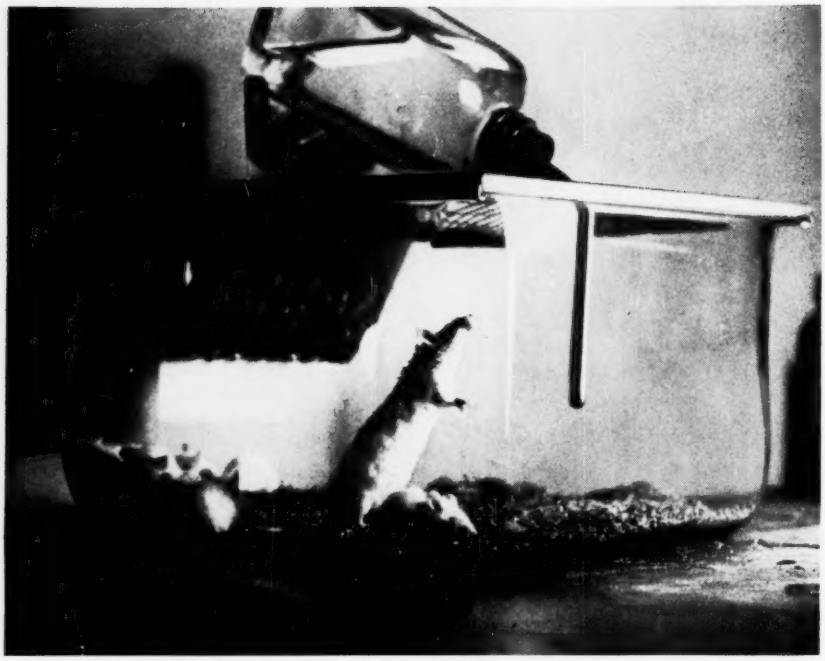

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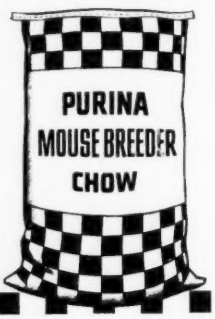

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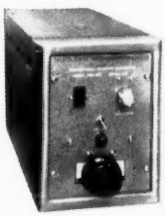

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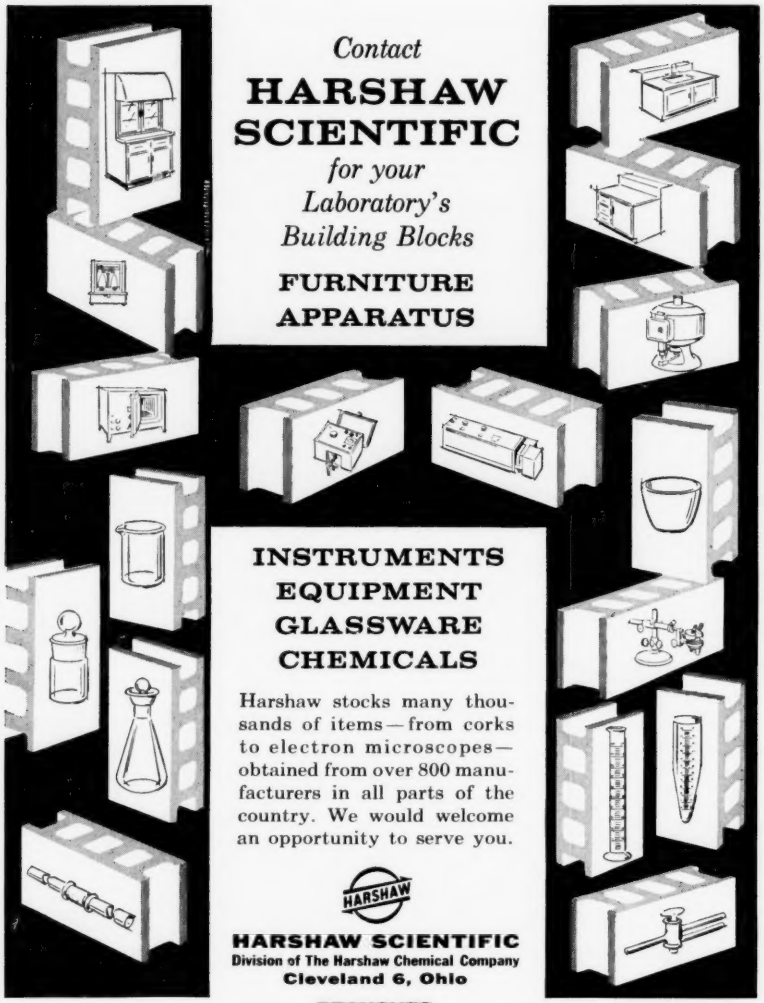

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